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PART IV

ENVIRONMENTAL PROTECTION AGENCY

TOXIC SUBSTANCES CONTROL

Discussion of Premanufacture Testing Policy and Technical Issues; Request for Comment

[6560-01-M]

ENVIRONMENTAL PROTECTION AGENCY

[OTS-050003; FRL-1069-1]

TOXIC SUBSTANCES CONTROL ACT PREMANUFACTURE TESTING OF NEW CHEMICAL SUBSTANCES

Guidance for Premanufacture Testing: Discussion of Policy Issues, Alternative Approaches, and Test Methods

AGENCY: Environmental Protection Agency.

ACTION: Discussion of policy and technical issues; request for public comment.

SUMMARY: Section 5 of the Toxic Substances Control Act (TSCA) requires prior notice of intent to manufacture or import a new chemical substance for a commercial purpose or manufacture import, or process for commercial purposes a chemical substance for a significant new use. The premanufacture notice must contain information about the chemical substance that is known to or reasonably ascertainable by the person submitting the notice, including information derived from testing for health or environmental effects. This document contains a discussion of policy and technical issues concerning guidance for premanufacture testing of new chemical substances. It also contains a series of tests and methods which the Agency has tentatively identified as appropriate for testing certain properties and effects of chemical substances. It does not constitute a proposal of premanufacture testing guidance. Rather, the purpose of the publication is to solicit public comment to aid in the formulation of a proposal to be published at a later date.

DATES: Interested persons should comment on this document on or before (90 days after publication).

ADDRESS: Written comments should bear the document control number OTS-050003 and should be submitted to Ms. Joyce Barbour, Document Control Officer (TS-793), Office of Toxic Substances, U.S. Environmental Protection Agency, 401 M Street, S.W., Washington, D.C. 20460.

FOR FURTHER INFORMATION CONTACT:

Mr. John B. Ritch, Director, Industry Assistance Office (TS-799), Office of Toxic Substances, Environmental Protection Agency, 401 M Street, S.W., Washington, D.C. 20460, 800-424-9065 toll free; in Washington, D.C. call 554-1404.

SUPPLEMENTARY INFORMATION: This document consists of two major parts. The first part (DISCUSSION OF ISSUES) contains a discussion of a number of policy and technical issues related to the creation of guidance for premanufacture testing of new chemical substances. EPA solicits comment on all issues identified in this part as well as any other issues related to premanufacture testing of chemical substances.

The second part (REFERENCE TESTS AND METHODS) contains a series of "reference tests" for eliciting information about the fate, health effects, and environmental effects of chemical substances. This part also contains, for each test, a discussion of its information objectives, the rationale for its inclusion as a "reference test", a detailed description of recommended test methods, and appropriate references. EPA solicits comments on the choice of tests and methods. A "Draft Technical Support Document" containing more detail about the tests and methods is available. It can be requested from Mr. John B. Ritch, Director, Industry Assistance Office (TS-799), Office of Toxic Substances, Environmental Protection Agency, 401 M Street, SW., Washington, D.C. 20460, 800-424-9065 toll free, in Washington, D.C. call 554-1404. Any person who intends to comment on tests and methods should review the relevant parts of the "Draft Technical Support Document."

Dated: February 20, 1979.

STEVEN D. JELLINEK,
Assistant Administrator for Toxic Substances.

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I. Discussion of Issues

A. SUMMARY AND REQUEST FOR COMMENTS

EPA believes that guidelines for premanufacture testing can substantially benefit the Section 5 premanufacture notice program and intends to propose such guidelines later this year.

The guidelines could be constructed in several different ways. The various approaches would differ in the degree of specificity of the guidance concerning premanufacture testing decisions. Of these different approaches, EPA's present long term goal is to publish a series of recommended tests and methods along with relatively detailed guidance concerning the need for testing for certain effects in given situations.

However, the inherent diversity of new chemical substances and exposure/use situations subject to the premanufacture notice program has thus far hampered EPA's attempts to construct detailed guidance. Therefore, EPA presently anticipates publishing guidelines which will be relatively general in nature. As the Agency gains experience with the premanufacture notice program, guidance concerning testing decisions can be made more detailed.

EPA is soliciting comment on the wisdom of both its long-term and short-term goals. In addition, the Agency solicits comment on how premanufacture testing guidelines can be constructed to encourage efficient testing of new chemical substances so that the maximum amount of useful information can be gained at least expense.

Specifically, the Agency would like to direct the attention of commentors to the following:

(1) Should guidance concerning the extent of testing and construction of testing systems be general or detailed? Is detailed guidance an obtainable objective?

(2) How can premanufacture testing guidance best be constructed to encourage cost-efficient testing?

(3) Should EPA adopt a base set approach to testing guidelines? Should the base set be "comprehensive" or "flexible," as those terms are defined in Part I? In either case, what tests should appear in a base set?

(4) Should EPA's testing guidelines relate the type or extent of recommended testing to projected production levels? To predicted market value or profitability?

(5) Should testing guidelines for new chemical substances address certain environmental or health effects, but not others? Should decisions not to address a particular effect be based on the adequacy of available test methods? The cost of testing for the effect? A judgment concerning the seriousness of the effect? Other criteria? Which effects should testing guidelines address?

(6) For what health and environmental effects do valid screening tests exist? For each screening test, what criteria should guide a decision to perform additional testing?

(7) In terms of guidelines for health effects testing, how should potential exposure to the new chemical substance relate to the type and extent of recommended testing? Can and should testing decision criteria be based on anticipated exposure? How should such criteria be constructed? For example, should they consider both extent of exposure (e.g., in a consumer product) and also intensity of exposure (e.g., plant workers)?

(8) How should testing guidelines address the question of the form of a substance which should be tested (e.g., pure substance, technical grade, formulation)? What are the cost implications of testing these different forms of a chemical substance?

(9) How should the cost of testing affect the structure of testing guidelines? For example, should the sequencing of tests in a tiered testing structure be determined in whole or in part by the relative cost of testing for various effects?

(10) What measures are available and desirable for reducing the economic impact of premanufacture testing consisting with EPA's information needs for assessing the risk of new chemical substances?

(11) Are there unpublished data that would improve EPA's and industry's ability to perform structure-activity analysis to determine whether testing for a particular effect is needed? EPA requests that any such data be made available to the Agency.

(12) What type of tests do companies now perform on new chemicals (known or suspected to be toxic; of unknown toxicity, etc.) and what type of risk assessments to they perform? What costs do companies experience in performing such tests?

(13) What kind of information (besides their own tests data) do companies have available on manufactured chemicals?

(14) Which specific tests do companies find useful for testing chemicals of various classes (or of various degrees of known toxicity)?

(15) In the context of Part II of this notice, REFERENCE TESTS AND METHODS, EPA solicits comments on the technical merits of the tests and methods set forth.

(16) Are the tests and methods described in Part II cost effective approaches for testing for the various effects? In what ways can the tests described in Part II be modified to decrease cost without substantially sacrificing scientific validity?

B. SUMMARY OF RELEVANT PROVISIONS OF TSCA

This section briefly summarizes relevant TSCA provisions in order to show how the section 5 premanufacture notification program relates to other information gathering and regulatory authorities.

Section 5(a) of TSCA requires premanufacture notification of intent to manufacture or import a new chemical substance for a commercial purpose. Section 5(d) requires that this notification include certain types of information, including studies of health and environmental risks.

Section 5(e) provides authority to limit or prohibit the manufacture of a new chemical substance pending the development of adequate information for an assessment of risk in certain circumstances. Section 5(f) provides authority for immediate regulation of a new chemical substance by administrative order in certain circumstances.

Section 4 of TSCA provides general authority to require testing of specific chemicals or categories of chemicals according to test standards promulgated by EPA. New chemicals which are subject to a section 4 test rule must satisfy the testing requirements before notice of intent to manufacture is submitted pursuant to section 5. Section 4(g) provides a mechanism whereby a person subject to the section 5 notice requirement may petition EPA to prescribe test standards for the development of test data.

Section 8 provides authority to require the maintenance of records and

the reporting of health and safety studies, information indicating the presence of substantial risk, and allegations concerning significant adverse effects on health or the environment.

Sections 6 and 7 provide general regulatory authority over chemical substances. Section 6 authorizes regulation by rule. Regulatory options range from labelling requirements to prohibition of manufacture, distribution, use or disposal. Section 7 authorizes EPA to commence civil action in district court for seizure of, or relief concerning, imminently hazardous chemical substances.

C. BACKGROUND

The Environmental Protection Agency (EPA) recently proposed rules and reporting forms to govern the submission of premanufacture notices for new chemical substances pursuant to section 5 of TSCA (40 CFR Part 720; 44 FR 2242; January 10, 1979). Section 5 of TSCA requires that any person who intends to manufacture or import a new chemical substance for a nonexempt commercial purpose in the United States, or manufacture, import or process for commerical purposes a chemical substance for a significant new use, must notify EPA at least ninety days in advance and provide certain information about the substance. This information will be used by EPA in performing risk assessments of new chemical substances proposed to be manufactured. The rules and forms referred to above propose the content and format of premanufacture notices. TSCA § 5(d)(1)(B) requires each notice to contain "any test data in the possession or control of the person giving such notice which are related to the effect of any manufacture, processing, distribution in commerce, use, or disposal of such substance or any article containing such substance, or of any combination of such activities, on health or the environment." Proposed § 720.23 would implement that requirement. The Agency has not yet proposed regulations for notification of significant new uses of chemical substances.

Because section 5 provides for submission of existing test data to EPA and EPA's use of these data to assess risk, the Agency plans to publish guidelines for new chemical testing as an aid to the development and submission of such data in premanufacture notices. The responsibility for deciding whether and how to test chemicals and assess risk rests initially on the manufacturer. Testing guidelines would be useful to the manufacturer because they would help him decide how much and what kinds of data are needed for a risk assessment.

EPA intends to propose testing guidelines later this year. However, during the course of developing testing guidelines, the Agency has encountered a number of policy and technical issues on which the Agency desires further discussion before guidelines are formally proposed. The purpose of this document is to solicit public comment on questions and issues related to guidelines for premanufacture testing of new chemical substances. In Part II of this document, a number of specific tests and methods are set forth for technical comment. The Agency will propose testing guidelines, including recommended tests and methods, after the review of public comments on this document.

D. STATUTORY FRAMEWORK FOR REGULATING CHEMICAL SUBSTANCES

The following discussion briefly describes TSCA as a regulatory mechanism and the contribution which testing guidelines might make to its effective implementation. Its purpose is to establish the role of the premanufacture notice process in the overall program of regulation created by TSCA.

1. *General Intent and Regulatory Structure.* A primary purpose of TSCA is "to assure that * * * innovation and commerce in * * * chemical substances and mixtures do not present an unreasonable risk of injury to health or the environment." (Section 2(b)(3)). The Environmental Protection Agency is charged with the responsibility of determining whether a new or existing chemical substance presents or may present an unreasonable risk of injury to health or the environment. Upon making such a determination, the Agency has available a range of regulatory authorities to eliminate or mitigate the risk.

The responsibility for developing the data and other information to enable EPA to perform risk assessments rests upon the manufacturers and processors of chemical substances. TSCA § 2(b)(1) expresses a policy that: "adequate data should be developed with respect to the effect of chemical substances and mixtures on health and the environment and that the development of such data should be the responsibility of those who manufacture and those who process such chemical substances and mixtures."

For purposes of regulation, TSCA distinguishes between (1) existing chemical substances and mixtures and (2) new chemical substances and significant new uses of exising chemical substances.

(a) *Existing Chemical Substances and Mixtures.* These are chemical substances included in the inventory that EPA is required to publish pursuant to section 8(b) of TSCA. At this time, the Agency is compiling this inventory and intends to publish it in May, 1979.

Existing chemicals can be regulated under several sections of TSCA, depending on the type and degree of risk to health or the environment which they are found to present. Regulatory options range from seizure of imminently hazardous substances, to prohibition or limitation of manufacture, use or disposal, to hazard warning labeling requirements.

EPA also has broad authority to acquire information concerning possible hazardous effects of chemical substances. TSCA contains various authorities and requirements concerning the retention and submission of records and test data related to health and safety studies, reported adverse effects, and known substantial risks. In addition, section 4 of TSCA gives EPA direct authority to require health and safety testing of existing chemical substances. Under section 4, EPA is required, upon making certain findings concerning possible risk or exposure and the need for test data, to promulgate test rules applicable to specific chemical substances or categories of chemical substances. The necessary findings are (1) that the substance may present an unreasonable risk of injury to health or the environment or that the substance is or will be produced in substantial quantities and lead to substantial environmental release or significant human exposure, (2) that there are insufficient data upon which the effects of the substance on health or the environment can reasonably be determined or predicted, and (3) that testing of the substance is necessary to develop such data. A section 4 test rule requires that certain tests be carried out on the particular substance or on substances in the category and specifies the test methods and data reporting requirements. If the substance is already being manufactured or processed for commercial purposes, the test rule is to specify a time period within which the tests must be conducted. Information developed pursuant to a section 4 test rule can be used in deciding whether and how to regulate the manufacture, processing, distribution, use or disposal of the chemical substance.

EPA has broad latitude in defining "categories" of substances for which testing rules may be promulgated pursuant to section 4, but is expressly prohibited from defining a category solely on the basis of being "new chemical substances" (section 26(c)(2)). However, once a category is defined and a test rule promulgated under section 4, any new chemical substance in the category will be subject to the test rule, and the information developed in accordance with the rule must be reported in the premanufacture notice required by section 5 (TSCA section 5(b)(1); proposed 40 CFR 720.20(j)).

(b) *New Chemical Substances.* A "new" chemical substance is any substance not included in the section 8(b) inventory. The statutory requirements concerning premanufacture notification for new chemical substances will become effective thirty days after publication of the inventory. The Agency also has authority under section 5(a)(2) to determine, by rule, that a particular use of a chemical substance constitutes a significant new use. Upon such a determination, the manufacture, import, or processing for the significant new use of the chemical substance is subject to statutory requirements similar to those pertaining to a new substance. Specifically, TSCA prohibits manufacture or import of a new chemical substance for commercial purposes, or manufacture, import, or processing for commercial purposes of any chemical substance for a significant new use, unless notice of intent is submitted to the Administrator of EPA at least 90 days in advance. For the sake of brevity, the term "new chemical substances" will be used in this discussion to refer to both new substances and significant new uses of existing substances. Similarly, the term "manufacturer" will be used to refer to the person submitting the notice whether, by statutory context, that person is a manufacturer, processor, or importer.

The notice of intent to manufacture a new chemical substance must contain certain information about the substance. This includes test data in the possession or control of the submitter as well as a description of any other data concerning environmental or health effects which is known to or reasonably ascertainable by him. However, section 5 of TSCA does not authorize EPA to require the development of new information on health and environmental effects by premanufacture testing of new chemical substances (except insofar as a new substance is subject to a section 4 test rule for a defined category of substances). Section 5(e) of TSCA does authorize EPA to take action to prohibit or limit manufacture, distribution in commerce, use, or disposal of a new chemical substance pending development of additional information on its health or environmental effects. Such action can be taken when the information available to EPA during the premanufacture notice period is insufficient to permit a reasoned evaluation of the health and environmental effects of a new chemical substance and when, in addition, the Administrator finds that the substance may present an unreasonable risk of injury to health or the environment or that it will be produced in substantial quantities and result in substantial human exposure or environmental release. Once sufficient information has been submitted and evaluated, EPA can proceed, if appropriate, to regulate the chemical substance according to the various authorities in the statute.

In summary, although EPA does not have authority to require premanufacture testing of all new chemical substances under section 5, the Agency can act to prohibit or limit manufacture in certain cases pending development of adequate information for a premanufacture risk assessment. Under section 4, EPA can promulgate test rules applicable to specific chemicals or categories of chemicals, provided certain findings are made. Therefore, there are two ways in which section 4 relates to the premanufacture review program. First, a section 4 test rule applicable to a defined category of chemical substances would apply to any new chemical substances within the defined category. In such cases, TSCA requires that the tests specified in the section 4 test rule be carried out before notice of intent to manufacture the chemical substance is submitted under section 5. Second, promulgation of a section 4 test rule is one of several actions available to EPA at any future time should its review of a chemical substance under section 5, or information from any other source, indicate that such rule is warranted.

2. *EPA's Long Term Strategy.* EPA's long term strategy is to issue section 4 test rules applicable to specific categories of chemical substances which meet the criteria of section 4(a) concerning potential for unreasonable risk or significant exposure. These test rules will require appropriate premanufacture testing of new substances that fall into the defined categories. In the long run, EPA's goal is to issue test rules so that a substantial proportion of new chemical substances will receive adequate premanufacture testing. However, it will be several years before the Agency issues enough section 4 test rules to reach this goal. In the meanwhile, the Agency plans to publish section 5 testing guidelines. These testing guidelines will be generally consistent with test rules which will later be issued pursuant to section 4. Unlike section 4 test rules, however, adherence to section 5 testing guidelines will be voluntary. It will be the responsibility of the manufacturer to determine, for each particular chemical, what type and degree of testing he will carry out before submitting the required premanufacture notice. Eventually, as section 4 test rules become effective, they will supersede the section 5 testing guidelines as they apply to the categories of chemicals covered by the section 4 rules.

E. ROLE OF TESTING INFORMATION IN PREMANUFACTURE REVIEW PROGRAM

The purpose of the premanufacture review program is to provide an opportunity to assess the potential for risk to health or the environment presented by a new chemical substance before significant human exposure or environmental release occurs. When a manufacturer develops a new chemical substance and proposes to produce it for commercial purposes, he should identify and assess risks which the chemical may present to health or the environment and take appropriate steps to eliminate or reduce such risks. Assessing the potential for risk to health or the environment depends upon knowledge of the physical and chemical properties and biological activity of a chemical substance. Many chemical manufacturers routinely perform testing to gain information of this type. The information may lead to a decision not to manufacture a particular substance because of its associated risk. It may also indicate the need for special industrial hygiene practices or restrictions on distribution, use or disposal which the manufacturer may incorporate voluntarily.

1. *"Unreasonable Risk" Determination.* Under TSCA, EPA is responsible for assessing the risk to health or the environment presented by chemical substances and for taking any regulatory actions which are necessary to prevent unreasonable risks. The phrase "unreasonable risk" is not explicitly defined by TSCA. The Agency intends to balance the magnitude of risks and social benefits associated with a chemical substance. In doing this, EPA will consider the seriousness of the risk (including the nature, extent, and reversibility of the adverse effects), the availability of alternatives to the substance and their associated risks, and the benefits (economic and otherwise) which accrue to society from the production and use of the substance. EPA must also consider the relative implications of the range of possible regulatory actions. A stronger determination of risk will be needed to support a ban on manufacture than a labeling requirement, for example.

2. *Risk Assessment.* The question of whether a risk is "unreasonable" cannot be addressed until a determination is made regarding whether a risk exists. To determine whether a substance does, in fact, present a risk of injury to health or the environment requires consideration of two types of factors. First, there must be an analysis of the physical, chemical, and biological properties of the substance and its potential for "injury" to health or the environment. The term "injury" may include various toxic effects in humans and other living organisms, including acutely toxic effects such as

poisoning and irritation of sensitive tissues and chronic effects such as cancer, birth defects, genetic damage, and long term or cumulative damage to living organisms in the environment. It may also include a broad range of other adverse environmental effects such as stratospheric ozone depletion, atmospheric modification, and tainting/odor problems. The second type of risk assessment factor is related to the prospects for human exposure or environmental release such that the potential effects referred to above might be manifested in the short or long term.

Analysis of the first type of factor described above may be referred to as "hazard assessment." Analysis of the second type of factor may be termed "exposure assessment." The dual nature of risk assessment acknowledges the interrelationship between hazard and exposure in the concept of "risk." It recognizes the common sense observation that there is more risk associated with a hazardous substance used, for example, in a consumer product than there is with an equally hazardous substance confined to a reaction vessel. Conversely, it recognizes that a more thorough evaluation of hazard potential is warranted for a product of widespread distribution or release than for a substance with limited distribution or release.

3. *Information Needed for Risk Assessment.* The potential for exposure or release may be judged from consideration of such information as production volume, production methods, and the anticipated distribution, use, and disposal of the substance. Human exposure predictions may focus either on particular segments of the population (e.g., plant workers, infants) or on the general public. Environmental release predictions may focus on the environmental compartment (air, water, land) into which the release is made, the distribution of the release (widespread or at few places), the volume and frequency of release, and the probability that particular organisms may be exposed to the substance in the environment. Regarding both health and environmental risks, attention may be given to potential transport pathways, degradation products, persistence, and tendency to accumulate.

Hazard assessment is the assessment of a substance's potential to cause deleterious effects in humans, other living organisms, or important nonliving environmental components. There are several possible sources of direct or inferential information on which a hazard assessment of a new chemical can be based. For example, most new chemicals are related structurally to existing chemicals. In some cases, the results of previous testing of "chemical cousins"—structural analogues—may indicate a potential for hazard for a new chemical substance.

As another example, substances which are defined as "new" for purposes of TSCA (and for which premanufacture notices thus are required) may in fact be existing chemicals for which test data exist. This could be the case for a TSCA "new chemical substance" which previously was produced and used abroad, one which was only manufactured in the United States before January 1, 1975, or which has previously been manufactured exclusively for a non-TSCA use (e.g., pesticides, drugs, or food additives). For such substances, some relevant data on hazardous effects may exist independent of any tests carried out for purposes of submitting a premanufacture notice. Such data would need to be evaluated for its contribution to risk assessment.

In many cases, however, there will be little or no information about the possible hazardous effects of a new chemical substance subject to premanufacture notice and what kind of information will be needed to support a risk assessment will depend upon the particular chemical substance and its intended use and exposure/release potential. Generally, several types of information may be needed:

(a) Information on the anticipated methods, locations, and volume of production, distribution, use and disposal may be needed to assess the potential human exposure and environmental release.

(b) Information on the substance's physical and chemical properties may be needed to evaluate potential transport pathways, degradation, and bioaccumulation.

(c) Information on potential effects on human health may be needed for a determination of health hazard.

(d) Information on potential effects on the environment may be needed for determination of both biological and non-biological environmental hazard.

Some information of the first type will probably have been accumulated by the manufacturer in the course of developing the chemical substance. Information of the other types, however, would generally be developed by testing the chemical substance in various ways. It is this type of testing that section 5 premanufacture testing guidelines will address.

EPA does not perceive the risk assessment process to be static or inflexible. Rather, it must be tailored to each new chemical substance. In some instances, the consideration of structure-activity data, previously available test data, and information on intended use and expected exposure may indicate that there is little potential that the substance would present a significant risk to health or the environment. In other cases, a more detailed and thorough assessment will be necessary to decide whether some form of regulation may be necessary to avoid creation of an unreasonable risk.

EPA is presently developing its risk assessment process for section 5 premanufacture review. For those new chemicals which will require a detailed assessment, it is apparent that the extent of the assessment may be constrained by the amount of time and data available for the assessment. Under TSCA, EPA has ninety days for premanufacture assessment, which can be extended for up to ninety days if the Administrator finds there is good cause for an extension. In addition, if EPA determines that insufficient information is available for a reasoned evaluation of health and environmental effects, and also makes certain findings related to the possibility of unreasonable risk or substantial environmental or human exposure, the Agency may proceed under section 5(e) to prohibit or limit manufacture pending development of the necessary information (proposed 40 CFR 720.36).

4. *Responsibility for Making Premanufacture Testing Decisions.* Premanufacture testing decisions are, in the first instance, the responsibility of the submitter. In order to clarify the role that voluntary testing guidelines might play in helping the manufacturer make these decisions, it is important to discuss the types of premanufacture decisions the manufacturer must make.

A manufacturer's premanufacture testing decisions can be divided into three groups. It is important to distinguish between these types of decisions, because various issues related to EPA's development of testing guidelines are closely related to the extent to which a particular guidelines approach does or does not lend support to a particular type of decision.

(a) *Whether to Test.* The first question the manufacturer must address is whether he has enough information about his substance to assess risks and make informed decisions concerning manufacture. To some extent, this decision will depend upon his perception of EPA's risk assessment process and information needs. If he decides that more information is needed, he must also decide whether testing is the best way to obtain the needed information. His decision whether to undertake testing may also depend upon factors such as his willingness or ability to pay the cost of the testing.

(b) *Type and Extent of Testing.* A closely related decision will involve determining the properties or effects about which more information is needed and deciding how much testing will be needed to achieve the desired results; in short, the manufacturer

must determine the amount of testing he will undertake. His decision will presumably be influenced by a number of factors, a few of which would include:

- The particular properties or effects which he feels are important for assessing potential hazard;
- The ways in which he anticipates the substance will be manufactured, processed, transported, used, and disposed;
- His ability or willingness to incur the expense of testing; and
- His perception of the amount and type of information EPA will need to have.

Having decided that he wishes to obtain more information about certain properties and effects, he must design a test plan which is responsive to these needs. The test plan should be designed to yield the needed information in a reliable and cost-efficient manner. He may wish to defer decisions concerning some tests until others have been carried out. If so, he may establish prior decision criteria for making that judgment. As discussed below, there are a variety of ways in which test plans could be constucted. The manufacturer must design a test plan to fit his particular needs.

(c) *How to Test.* As part of the design of his test plan, the submitter must choose the particular tests which he will perform. In addition, he must decide upon specific methods for carrying out each test. These decisions will depend, in part, on his perception of the scientific validity of various tests and methods, their relative costs, and his perception of whether EPA prefers certain tests over others for any given property or effect.

Once the above decisions have been made and the test plan has been executed, the manufacturer again faces the first type of decision in terms of the adequacy of information then in his possesion. Eventually, he must decide either that (1) the risks associated with the substance are such that he should proceed no further in its development, (2) that the economic potential of the chemical does not warrant incurring any further expense, or (3) that he will submit a premanufacture notice reflecting the information at hand. The information derived from testing may lead him to develop safeguards in manufacture, processing, distribution, use or disposal to reduce exposure and therefore risks he has identified.

F. STRUCTURE AND FUNCTION OF TESTING GUIDELINES

1. *Alternative Approaches.* There are four general approaches to testing guidelines available to EPA. In the discussion that follows, each is identified and its advantages and disadvantages discussed. The discussion of alternatives is followed by a discussion of different approaches which could be taken in developing guidance consistant with EPA's presently preferred alternative.

Alternative 1: No action.

EPA could say nothing about premanufacture testing, providing no comment on any of the three types of testing decisions mentioned above. An advantage of this approach to EPA would be that it is the easiest course to take, freeing staff resources for work on other current TSCA-related activities. Some manufacturers have indicated a preference for this alternative, stating a concern that even voluntary testing guidelines will be used as a "checklist" when EPA is determining the adequacy of submitted information for a risk assessment. If "no action" were the course followed, the manufacturer would make his own decisions without any guidance from EPA. No explicit or implicit presumptions would be established by EPA regarding the desirability of testing in given situations or the applicability of any particular test.

However, this alternative would also present some disadvantages to both EPA and the manufacturer. The manfacturer would make his decision in ignorance of EPA's expectations regarding the information needed to carry out its assessment of risk. As a result, the Agency might determine that a greater number of premanufacture notices contain insufficient information. EPA might need to proceed more often under section 5(e) to regulate substances pending development of information. This could result in unnecessary delays in manufacturers' production schedules, litigation, and the diversion of EPA's technical and legal resources. In addition, nonuniformity of testing methods would be maximized. EPA's risk assessment of a chemical substance would have to focus as much on evaluating testing methodology as evaluating results. Maintaining an equitable and evenhanded review process would be more difficult.

Alternative 2: List of Tests and Recommended Methods.

Another alternative would be to publish a list of tests which are appropriate for specific properties and effects and recommended methods for carrying out each test. The basic thrust of this alternative would be to say, "If you choose to test for a particular effect or property, here is a test which EPA believes is appropriate and here is a recommended method for conducting the test."

This alternative would offer several advantages to both EPA and the manufacturer. First, EPA's task in preparing guidelines would be relatively easy compared to other alternatives. The Agency's role would be to identify state-of-the-art tests for each effect of concern and choose one or more recommended methods. In terms of the types of premanufacture testing decisions outlines above, it would provide guidance concerning only the third type, "how to test."

The listed tests and recommended methods would not be required. They would be suggestions, and the manufacturer would be free to test for any particular effect in any way he wished. However, to the extent that the recommendations were followed, EPA's (and in many cases the manufacturer's0 risk assessment process would be made somewhat easier. EPA's attention could be focused on the results of the recommended tests rather than on the scientific validity of a variety of other tests and methods. This would allow a more efficient, thorough and evenhanded risk assessment. In the long run, the creation of a comparative data base on the properties and effects of chemicals, derived from uniform test methods, would prove valuable. Structure-activity correlations might be more readily identified, reducing the future need for certain types of tests. Some types of effects and tests might prove predictive of others, thereby decreasing the need for testing. In the long run, the development of a consistent data base could only make testing and assessment more cost-efficient.

Testing guidelines constructed in this way would have several disadvantages, however. They would provide no guidance at all for decisions about "whether to test" and "type and extent of testing." Nor would they be of much help in deciding how to sequence tests so that preliminary tests would indicate the need for further tests. The absence of such additional guidance from EPA could result in uneven premanufacture testing. In order to guard against delays that might occur when EPA acts to obtain more information, some manufacturers might carry out more testing than EPA would think necessary. Others might underestimate EPA's information needs and, therefore, encounter problems which could have been avoided. Either way, the result would be inefficiency in the premanufacture testing program. The results might be more adverse for small companies for whom testing is a proportionately larger burden and marketing delays a greater threat. Decisions might be made to stop development of chemicals which, had there been some guidance from EPA concerning its information needs, might have been marketed. This could have a needless ad-

verse impact on the pace of chemical innovation.

Alternatively, EPA could provide some form of guidance concerning the applicability of specific tests to different types of chemicals and exposure/use situations. In theory, this guidance could be quite general or quite detailed. For purposes of comparison, this discussion will identify two alternative forms of guidance at opposite ends of the spectrum—relatively general guidance (Alternative 3) and relatively detailed guidance (Alternative 4). It should be understood, however, that these alternatives differ more in degree than in kind. A guidelines package could be general in some respects and detailed in others.

Alternative 3: List of Tests with General Guidance Concerning their Use.

EPA could publish general guidance concerning the applicability of the recommended tests and methods in particular circumstances. The applicability guidance would address "whether to test" and "type and extent of testing" decisions, but it would be relatively general in nature. Thus, it might describe EPA's view of the relationship between potential hazards and potential exposure in making risk assessments. It might indicate in general terms EPA's perceived information needs regarding different types of chemicals and exposure/use situations. It might also discuss ways in which a testing plan could be structured—that is, ways in which tests might be sequenced and, in general, how a manufacturer might decide the kinds of results which indicate the need for certain kinds of additional tests.

Such an approach would have the advantages mentioned above in Alternative 2. In addition, it would remove some (but not all) of the uncertainty regarding EPA's risk assessment process and information needs. To the extent that the guidance adequately reflected EPA's risk assessment process and was followed by the manufacturer, it would result in testing that would be relatively more appropriate for EPA's risk assessment needs. Therefore, testing would be more efficient and there would be fewer notices with information which, in EPA's view, was insufficient. Avoidable delays would be lessened.

On the other hand, general guidance would not completely dispose of the "whether to test" and "type and extent of testing" decisions. If the manufacturer wished to follow EPA's advise, he would need to relate the general guidance to his particular needs. Depending upon his interpretation of the general guidance, he might perform more or less testing than actually warranted.

Alternative 4: Recommended Tests With Detailed Guidance Concerning Their Use.

This alternative would incorporate the recommended tests and methods in Alternative 2 but would contain relatively detailed guidance concerning "whether to test" and "type and extent of testing." There are a number of ways in which detailed testing guidance could be constructed, ranging from a single "base set" of recommended tests to tiered testing schemes based on various scientific and policy considerations. Some of these approaches to detailed guidance are discussed in a later section.

Detailed guidance might specify conditions under which certain types of testing would be warranted. It might also specify quantitative criteria for interpreting test results and determining the need for additional testing.

An advantage of detailed guidance is that it would be more help in resolving "whether to test" and "type and extent of testing" questions. The manufacturer would have stronger assurance that, if he followed EPA's guidance, the information developed would more likely be appropriate for EPA's risk assessment. This alternative would provide more explicit guidance, would probably increase the efficiency and appropriateness of testing, and would minimize delays occasioned by the submission of inadequate information.

A principle drawback is the difficulty of implementing this alternative. Relatively detailed guidance concerning whether and how much to test is difficult to develop unless the particular type of chemical and exposure/use information is known. Actual "whether to test", "type and extent of testing" and test plan design decisions can best be made in the context of particular chemical substances.

Another drawback is that more detailed decision criteria might be perceived as being regulatory requirements, even though issued in the form of voluntary guidelines. They might thereby limit the manufacture's perceived range of choice. Submitters might feel that they could not deviate from EPA's recommendations.

Alternatives 3 and 4 differ in the degree of detail in which guidance concerning "whether to test" and "type and extent of testing" would be stated. In fact, these two alternatives are presented in this way to emphasize the extremes in such guidance for purposes of discussion. These alternatives represent the ends of a spectrum, and the discussion attempts to point out the consequences of guidelines of both extremes.

As the Agency has explored the development of testing guidelines, some individuals have urged the adoption of very general guidance on "whether to test" and "type and extent of testing" questions. They argue that manufactures are experienced at testing and that their scientific judgement should not be constrained or directed by testing guidelines. Others have encouraged the publication of detailed guidance. They point out that, since EPA will assess potential risks presented by new chemical substances, the Agency should disclose its position on testing and the use of test information for assessment in as much detail as possible.

2. *EPA's Long-Term and Short-Term Goals.* EPA's long-term goal concerning premanufacture testing guidelines is to provide recommended tests and detailed guidance on how to use them in a premanufacture testing program. The Agency has been working actively to develop such guidance by the time the premanufacture notice program begins in Spring, 1979. However, it is doubtful that detailed guidance can be fully developed by that time.

Because EPA believes there is a need to have testing guidelines in place as soon as possible, the Agency is considering proposing more general guidelines as an interim approach. More detailed guidance can be published as the Agency gains knowledge about existing testing programs and further develops its risk assessment information needs. EPA is considering developing a guidelines package which would contain recommended tests and methods along with general guidance on "whether to test" and "type and extent of testing" issues. The remainder of Part I of this document discusses the development of testing guidelines addressing these issues. Public comment on these issues will help EPA fashion its proposal of interim testing guidelines, as well as its longer term development of more detailed guidelines. Part II presents a series of tests and methods which the Agency has tentatively identified as appropriate for inclusion in a testing guidelines package. For any particular effect for which decision to test has been made, these "reference tests" would provide guidance on "how to test" questions.

G. DEVELOPMENT OF TESTING GUIDELINES

For the past several months, EPA has made an intensive effort to develop premanufacture testing guidelines which will support the three types of testing decisions described above. During this time, the Agency has received comments and recommendations from a number of trade associations, public interest groups, and other groups and individuals interested in testing issues. For reasons discussed below, EPA has decided not to endorse at this time any of the testing schemes proposed by such groups and

individuals. The following discussion describes the problems and prospects for testing guidelines and identifies related policy and technical issues which must be resolved. The Agency invites public comment on how testing guidelines could best be constructed in order to facilitate the effective and efficient implementation of TSCA.

As noted above, EPA has tentatively identified a number of tests and methods which it feels are appropriate for testing for certain physical and chemical properties and health and environmental effects. The tests and methods which the Agency has selected are set forth in Part II of this document. The tests are intended to function as "reference tests"—that is, recommended tests to which a person may refer once a decision has been made to test for a particular effect or property. These recommended tests were selected because they are standardized tests in common use or because they generally represent the "state of the art." In those cases, futher validation of a particular test is needed. In those cases, the candidate test is judged to be the best available at this time.

More difficult than selecting tests and methods is the problem of providing guidance concerning their use—that is, guidance concerning "whether to test" and "type and extent of testing" decisions. Discussed below are some of the factors which must be considered in developing testing guidance and some alternative ways in which guidance might be constructed.

1. *Context of Testing Decisions.* Any attempt to devise premanufacture testing guidance concerning "whether to test" and "type and extent of testing" is constrained by an unavoidable problem. The purpose of premanufacture testing guidelines, as discussed above, is to assist the manufacturer's decision making process and to encourage testing which is compatible with EPA's performance of risk assessments. The manufacturer makes his testing decisions in the context of particular chemical substances. However, the entire universe of new chemical substances is subject to section 5, so to be comprehensive, premanufacture testing guidelines would have to address the great variety of future chemicals and use and exposure patterns. It is very difficult to develop specific guidance with recommended tests to apply to all such substances and situations. In the case of a chemical substance that would be widespread, mobile, and persistent and is closely related to substances of known toxicity, thorough testing by diverse means would be needed to adequately evaluate risks. At the other extreme, less testing might be needed for a low exposure substance (e.g., one to be reliably contained) unless and until its use should change.

If, as EPA expects, the majority of chemicals subject to premanufacture notice fall somewhere in the middle, it would be neither practical nor prudent to expect the full range of testing for every chemical. A point of diminishing returns can be reached where the additional social benefit of testing is offset by the cost of testing and the possibility of inhibiting the development of a socially beneficial product. EPA cannot identify this point in advance for all yet-unknown chemicals. Rather, it must be evaluated by considering previously available information on toxicity and exposure, societal benefits of producing the chemical, the value society places on relative certainty about the safety of a chemical, and the costs of additional testing. These factors are variable.

2. *Approaches to Testing Guidelines.* There are several approaches to the development of testing guidelines which EPA could adopt in pursuit of its goal of detailed guidance. They can be judged according to several different criteria, including economic impact, scientific validity, and practicability. In the final analysis, there is an implicit tension between society's need for assurance that the chemicals distributed in commerce are safe and the social benefits of chemical innovation. In some cases, the cost of developing sufficient information to be reasonably certain of the health or environmental hazard presented by a chemical might preclude marketing of a new chemical substance. Because many new chemicals will probably present little risk and yet promise social benefit, premanufacture testing must be carried out in a flexible and reasonable manner. Premanufacture testing must be adequate, however, to detect and assess new chemical substances which may present an unreasonable risk of injury to health or the environment.

(a) *Comprehensive base set.* One approach theoretically available to EPA is that of a single "comprehensive base set." EPA defines a comprehensive base set as a set of tests to produce data which EPA would presume to be necessary for every new chemical substance. The tests could address a wide range of effects and physical/chemical properties. The base set would therefore be "comprehensive" in terms of both effects covered and applicability to chemical substances. An ideal comprehensive base set would not be unreasonably expensive, would apply to all chemicals, and would provide enough information for at least a preliminary risk assessment.

A distinguishing characteristic of the comprehensive base set approach is the presumption that a certain set of data is both *necessary* for every new chemical substance and *sufficient* for assessment purposes. The comprehensive base set approach would thus provide a maximum amount of guidance on the question of whether and how much to test a new substance. A manufacturer who carried out every test in the base set could be reasonably assured of fulfilling EPA's assessment information needs. On the other hand, an inflexible comprehensive base set could be inefficient should it include tests which are not appropriate for a significant number of substances.

At this time, EPA believes that the single comprehensive base set approach is not a viable alternative for premanufacture testing guidelines. The diversity of chemical substances subject to the premanufacture notice requirements of section 5 appears to preclude any single set of data which would be both necessary and sufficient for assessing every new substance. Another consideration is cost. A set of tests which would provide sufficient information for a risk assessment for all chemicals would be sufficiently costly to preclude further development of many chemical substances. The difficult tradeoff between the need for information and the cost of testing would still have to be addressed without reference to a particular chemical substance and exposure/use situation.

(b) *Categorical base set.* Another alternative which EPA might pursue is to define categories of chemicals and produce a base set of tests for each category. Chemical categories might be defined based upon chemical structure, intended use, production level, or other factors or combinations of factors.

This approach would avoid some of the problems associated with the diversity of chemical substances. More specific guidance could be written than would be feasible with a single comprehensive base set as discussed above.

EPA would need considerable time to develop categorical base sets. EPA solicits comments on the utility of developing a series of categorical base sets over a period of several years. In particular, the Agency would appreciate comments on how the categorical base set approach could be implemented and whether the contributions to the premanufacture review program would outweigh the resources required to develop the various base sets. The Agency also seeks comment on how it could define categories of chemicals and the types of tests that would be appropriate for each category.

(c) *Flexible base set.* Some of the problems with both the comprehensive and categorical base set approaches result from the attempt to

apply a set of tests to an array of chemical substances to produce, for each substance, sufficient information for a preliminary risk assessment. Ways might be devised, however, to incorporate flexibility into the base set approach. This would allow testing for a particular substance to be "tailored" to its characteristics and, perhaps, its expected exposure patterns. One such approach would be to establish a base set of tests while including a series of exceptions that delete certain tests in certain situations. For example, EPA could advise the submitter to delete tests for physical/chemical properties which were clearly inappropriate for his substance but maintain the presumption that all the health and environmental effects tests be performed.

Further, exceptions could be based on exposure and use considerations. For example, EPA might advise that environmental effects testing is not needed for substances for which the chance of significant environmental release is slight.

Another approach would be to define a minimum base set of tests applicable to all substances and provide guidance for adding other tests appropriate to the particular case. This approach is discussed below as a "tiered testing" approach.

The flexible base set approach would contain more flexibility than the comprehensive or categorical base set approaches. As more flexibility is built in, however, the advantages of detailed guidance are lost. The probability increases that insufficient or inappropriate information may be developed.

EPA solicits comment on the flexible base set approach to testing guidelines. Specifically, the agency would like to receive comments concerning what tests might be placed in the base set and how guidance concerning exceptions might be constructed.

(d) *Tiered Testing Systems*—(1) *General.* In a tiered testing scheme, tests or groups of tests are arranged in a hierachical structure. Testing begins with tests in the lowest tier and proceeds as appropriate to higher tiers. Decisions on whether to go on to higher tier tests or to "exit" from the testing scheme are made according to decision rules or criteria. These decision rules may be general and qualitative or specific and quantitiative. The latter are often referred to as "triggers."

In theory, a tiered testing scheme contains "screening tests" in the first tier, followed by other predictive and confirmative tests in higher tiers. The screening tests are designed to disclose the potential that the chemical may exert a toxic effect. Positive results from screening tests indicate the need to carry out more elaborate tests to confirm the presence or absence of the toxic effects or to further characterize or improve the reliability of the original prediction. The attractiveness of the tiered structure is that is is presumably not necessary to carry out the more expensive confirmative tests in cases where the screening tests yield negative results. Thus, in theory, a tiered testing scheme can promote cost-efficient testing.

The tests and decision rules in a tiered system can be arranged in a variety of ways, depending on the purpose of a particular testing scheme and the degree of conservatism to be built into it. In other words, a tiered system can be constructed to yield data for a comprehensive risk assessment or a preliminary risk assessment. It can address a wide range of substances and effects, or only a few.

There are a number of considerations that can influence the selection and sequencing of tests within a tiered system. For example, tests can be arranged in such a way that each effect of concern, such as acute toxicity, chronic toxicity, cancer or birth defects, has equal priority, and all effects receive adequate testing. If it is scientifically justifiable, a tiered sequence could be developed for each effect with screening tests in the first tier, and other predictive and confirmative tests in higher tiers. The latter tests would be more extensive and more costly, and would require more time to complete. They would need to be performed only when positive results from earlier tests indicated a potential for the particular effect of concern.

For certain important health and environmental effects (e.g., animal oncogenicity and chronic toxicity) there may be no validated short-term or screening tests. Therefore, tiering based on scientific criteria is not possible for all properties and effects. The lack of short-term or screening tests means that it would be necessary to perform expensive tests if one wishes to test for all significant effects. As a result the first tier of tests would be very expensive. Thus, one of the major theoretical advantages of a tiered testing system could be diminished in practice if tests for all important effects are included.

A decision to include only short-term or screening tests in the first tier, on the other hand, would mean that no testing would be conducted for some effects. Negative test results concerning those effects for which screening tests are available would mean that higher tiers would not be reached. Thus, effects represented only in the higher tiers would go untested in such cases. EPA prefers not to ignore certain toxic effects simply because inexpensive short-term screening tests are not available.

One possible tiered testing scheme is similar to, and in effect builds upon, the flexible base set approach discussed above. EPA could identify a minimum base set of tests to provide data which would be presumed necessary for every chemical substance. This set of tests, which could be designated "Tier O," might relate to important physical or chemical properies, such as vapor pressure and octanol/water partition coefficient. The Tier O tests could be used to determine which types of Tier 1 (short-term and screening) tests would be appropriate for particular chemical substances.

A tiered testing scheme could also be constructed based on economic as well as scientific considerations. Such a scheme would have the explicit objective that less expensive tests would be performed in the first tier and more expensive tests in higher tiers. This approach would result in a less costly testing program, because only the less expensive tests would be carried out routinely. More expensive tests would be carried out only when the less expensive tests yielded postitive results, or (for effects for which there are no short-term or screening tests) when other criteria for testing are met. These latter criteria could include production volume, company size, chemical profitability, and so forth.

There are two constraints associated with this approach which limit its utility for scientific risk assessment. First, since the sequencing of tests would be determined in part by their relative costs and not solely by the reliability of the first tier tests as screens for the higher tier effects, questions of the scientific adequacy of the resulting data might arise. Second, the consequence of such an approach would automatically be that effects for which inexpensive tests exist would be given priority over other effects for which there are no such tests.

Though attractive from an economic and pragmatic point of view, such a testing system may not adequately protect society from hazardous chemical substances. For example, it is arguable that long-term chronic health effects should be of greater concern to EPA under section 5 than short term acute effects. If so, performance of chronic studies should not depend on the results of short term tests which are not scientifically related to the evaluation of chronic effects.

A tiered testing system should include criteria following each tier to guide decisions concerning whether to go on to higher tiers or terminate testing. These decision criteria can be quantitative or qualitative, rigid or flexible. Various suggested testing schemes, for example, have incorpo-

rated decision criteria based upon exposure or environmental release potential, such as quantitative or qualitative estimates of production volume, discharge volume, specific uses, or estimated environmental concentrations. Other decision criteria have been suggested on the basis of indicators of environmental persistence or bioconcentration potential, such as volatility or sorption loss rates, hydrolytic half-life, BOD/COD ratios, water solubility, or octanol-water partition coefficients. Further, most schemes employ one or more decision criteria related to acute animal toxicity, in the form of LD_{50} or LC_{50} values, relationships of static to flow-through bioassay data, or other parameters. It is important to recognize that decision criteria, like the tier structure itself, can be based on both scientific and policy consideration. For example, triggers can be established that make it relatively difficult or relatively easy for a substance to exit from the testing system. Establishing decision criteria which are extreme in either direction defeats much of the purpose of the tiered system. Therefore, the nature and sensitivity of decision criteria have a great deal to do both with the expense of testing a substance and the degree of certainty that the testing system will yield a low percentage of false negative and false positive results.

EPA could sequentially develop a series of tiered testing systems on the basis of categories of chemicals or use or exposure patterns. This approach would eliminate some of the difficulties inherent in addressing the universe of new chemical substances in a single testing guidelines package. It probably would permit more detailed guidance. However, more time and resources would probably be required to develop this approach.

(2) *Testing schemes recommended to EPA.* In the course of developing testing guidelines, EPA has considered a number of tiered testing schemes developed by various interested parties, such as the Conservation Foundation, National Academy of Sciences, European Economic Community, American Institute of Biological Sciences, American Society for Testing and Materials, and several chemical manufacturers. Though the schemes have been developed by diverse groups, they have some elements in common. Generally speaking, the recommended test schemes tend to contain basic physical and chemical properties and short-term acute toxicity tests in the lower tier, and long-term, chronic effects tests in higher tiers. The acute tests are often called "screening tests," though there is often little or no scientific support for the proposition that the results of the acute tests are predictive of certain chronic effects to be tested in the higher tiers. In some cases lower tier tests appear to be placed there primarily because they are less expensive.

Some of the recommended systems, though recognizing the need for decision criteria for progressing from one tier to the next, do not contain such criteria. In those that do contain criteria, the criteria often appear to be based on subjective judgments involving economic and methodoligical concerns as well as scientific considerations. Although such judgments may be necessary to arrive at usable decision criteria, the analysis on which they are based should be explicit so that the validity and implication of the criteria can be evaluated.

3. *The Cost of Testing.* It appears that many chemicals currently in commercial production are low volume, specialty chemicals. Preliminary information derived from the inventory of chemical substances developed by EPA under section 8(b) of the Act suggests that approximately 75 percent of all chemicals in production in the United States have annual production volumes of less than 100,000 pounds. Most of these chemicals have been developed for specific uses. They compete with numerous potential substitutes and face highly price sensitive demand. Presumably, many new chemical substances will share some of these features. For a typical new substance, initial production may be low and increase as the substance penetrates the market. Making conservative assumptions about sales prices, gross margins on sales, product lives, and start-up costs, it appears that for many new chemicals it may not be economically feasible to perform very much testing for health and environmental effects or chemical fate at the premanufacture stage. For some chemicals, the manufacturer might therefore decide to forego production of the chemical rather than incur the cost of testing it. It is thus possible that premanufacture testing sufficient to produce the information needed for a reasoned risk assessment could inhibit innovation in the chemical industry. A major objective of testing guidelines is to minimize this effect by focusing testing efforts on substances which, because of anticipated exposure or preliminary indication of toxicity, are more likely to present a risk to health or the environment.

Perhaps the most difficult situation arises when a new chemical substance is expected to be produced in small quantities but for a use that will expose large segments of the population. An example would be minor component of a widely used consumer product. In this situation, the probability of widespread human exposure would indicate the need for health effects testing. However, the anticipated low production volume might mean that the anticipated profits from the chemical would be insufficient to cover the costs of the necessary health tests. In this situation, the manufacture might decide to not attempt to market the substance.

Considering the premanufacture review program as a whole, it is difficult to predict accurately the actual degree to which chemical innovation might be inhibited by testing guidelines. It would depend on the cumulative individual decisions of many manufacturers developing a variety of chemicals for diverse uses.

It is reasonable to generalize, however, that detailed guidance will encourage more testing to be carried out. Due to the cost of testing when guidance indicates the need for it, some low market potential chemicals would not be produced. However, the public would gain greater certainty that those that are produced do not present and unreasonable risk to health or the environment. Conversely, more general guidance could result in less testing. Therefore, there would be more chemical innovation but less certainty concerning risk. Finding the proper balance between information needed for public assurance of safety and societal benefits from chemical innovation is a vital part of the long term successful implementation of TSCA.

TSCA addresses this implicit policy conflict which it creates. In the Statement of Policy in Section 2, TSCA states that "authority over chemical substances and mixtures should be exercised in such a manner as not to impede unduly or create unnecessary economic barriers to technological innovation while fulfilling the primary purpose of the Act to assure that such innovation and commerce in such chemical substances and mixtures do not present an unreasonable risk of injury to health or the environment." (Section 2(b)(3)).

In the context of the premanufacture notice program, EPA especially seeks public comment on how to balance the "primary purpose" of TSCA, to assure that new chemical substances do not present an unreasonable risk, with the admonition that the Agency not "unduly" impede innovation in the chemical industry. The Agency seeks comment on how premanufacture testing guidelines can be constructed to encourage the appropriate balance between the need for test data and the cost of obtaining them.

There are several approaches to the question of guidelines for premanufacture testing which could mitigate adverse effects on innovation. Perhaps the most straightforward is the use of

"production volume cutoffs" as decision criteria in a tiered scheme. With such an approach, EPA would advise manufacturers that it would expect little or no testing for substances which will be produced under certain volume limits. This approach would remove the economic burden of testing for many low volume chemicals. However, it would be based on the implicit assumption that low production volume equates with low exposure and therefore low risk. Under this approach, low production-high exposure chemicals would probably go untested even though they might be very hazardous. Also, the assumption that low production implies low exposure does not consider particular segments of the population, such as production or processing plant workers, whose exposure might be quite high. Given EPA's responsibility for considering all types of exposure, an assumption which implicitly "averages out" a production volume over the whole of society in deciding that exposure levels will be acceptable requires close scrutiny. Thus, there are issues associated with the production volume cut-off approach which weigh against its economic merits.

Another approach which might mitigate the overall economic impact of premanufacture testing guidelines involves the selection of particular "effects of concern". Under this approach, EPA would limit its testing recommendations to those effects the Agency deems to be of greatest importance in the review of notices for new substances. Cost estimates for testing the properties and effects with which EPA could be concerned range from a few hundreds or thousands of dollars for many of the chemical fate and ecological effects tests, to tens of thousands and even hundreds of thousands of dollars for some of the health effects tests. Biodegradation tests, for example, cost in the neighborhood of $5,000–$12,000 to perform, while subchronic health tests cost $100,000 or more and chronic toxicity and oncogenicity studies cost $300,000-$700,000. By focusing on certain effects, EPA would decrease the cost of testing for particular substances. However, the Agency would do so at the expense of obtaining information about many effects. Because some of the more expensive tests relate to human health effects, it might be difficult to implement the "effects of concern" alternative in such a way as to both reduce cost and protect important public health values.

A third way to mitigate the costs of testing would be to modify the conventional test method which would be included in a base or first tier set so as to reduce the overall cost of the set. For example, less expensive test organisms could be used in 90 or 180 day subchronic testing, or the number of recommended tests in a mutagenic/oncogenic screening battery could be reduced. A problem with this approach is the loss of predictive power that might result. EPA is actively pursuing this option by seeking ways to reduce the cost of particular tests while maintaining adequate scientific predictability.

Another alternative would be to adopt a "deferred testing" approach. For certain new substances which EPA determines will present little potential risk at initial production levels and intended uses, EPA could issue significant new use rules (SNURs) under section 5(a)(2). The SNUR for a substance could identify possible future production levels and uses for the substance. Should these conditions be realized, the substance would again be subject to section 5 review in terms of its new production level and uses. At this point, the section 5 review might indicate a need for certain additional testing. Because the chemical probably would have a greater market potential than when first manufactured, it should be better able to support the costs associated with additional testing. The general effect of using SNURs in this way would be to relate levels and costs of testing to the expanding market strength and profitability of new chemicals. Such a program could result in a minimum adverse effect on production of new chemicals while ensuring that those which are successful will be subject to further EPA review.

EPA does not prefer to allow small volume chemicals, regardless of their uses, to be produced without some testing. However, it is not the Agency's intent to encourage extensive testing of all new chemicals. EPA is aware that in some cases a relatively small amount of data of certain types will be sufficient to perform an adequate risk assessment, while in other cases data from a full range of tests, including chronic toxicity and oncogenicity tests, will be needed. As EPA gains experience in performing risk assessments under TSCA, the tradeoffs between the costs of generating information and the value of that information will become better understood and incorporated into guidelines and section 4 testing rule decisions. Further, testing technology has evolved rapidly in the recent past, and EPA expects that it will continue to do so. As new tests are developed and correlations among tests are documented, EPA will revise the premanufacture testing guidelines. The goal will be to maintain state of the art cost effectiveness and scientific validity as knowledge of testing technology advances.

Finally, EPA is aware that it is operating in an area of relative uncertainty in its efforts to develop testing guideline and incorporate them into the premanufacture review program. For a number of reasons, it is difficult to perform economic assessments of alternative approaches to testing guidelines. For example, the Agency does not know for a certainty how many new substances will be introduced each year; the types of chemicals they will be; how many of them will be small volume, low profit chemicals; or what current industry practices are with respect to health and environmental testing. Equally important, the true economic cost of a particular approach to testing guidelines depends upon the extent to which manufacturers follow EPA's recommendations, a factor about which EPA cannot obtain advance knowledge.

II. Reference Tests and Methods

As discussed in Part I above, EPA plans to propose testing guidelines to support implementation of the premanufacture notification provisions of section 5 of the Toxic Substances Control Act (TSCA). As a first step in developing testing guidelines, EPA has tentatively identified a number of "reference tests" for specific physical and chemical properties and health and environmental effects of chemical substances. The reference tests will advise a manufacturer who has decided to evaluate a particular property or effect which test or tests EPA feels are most appropriate. Each reference test will be accompanied by a detailed description of the preferred method for conducting the test.

Though adherence to the reference tests is voluntary, EPA believes that publishing recommended tests and methods will encourage uniformity of testing methodology. Uniformity of testing methodology is desirable because it would facilitate risk assessment. Use of a recommended test and method would assure acceptance of the test and method as valid. It would also allow EPA to focus its assessment on the results of tests rather than on evaluation of a variety of test methods.

The reference tests will address a broad range of properties and effects. The Agency does not foresee that all reference tests would be carried out on every new chemical substance. Rather, the reference tests will provide a list of recommended tests from which a manufacturer may, if he desires, choose tests which are appropriate for his test plan.

Also, EPA is participating in two major efforts to develop multiagency agreement upon test methods. The first of these involves the Interagency Regulatory Liaison Group (IRLG),

which consists of the Occupational Safety and Health Administration, the Consumer Product Safety Commission, the Food and Drug Administration, and EPA. This group is identifying test methods which would yield data suitable for submission to any program within the four agencies.

The second effort aimed at building agreement on testing methods is an international effort sponsored by the Organization for Economic Cooperation and Development (OECD). The OECD activity seeks to identify agreed-upon test methods in order to avoid duplicative testing.

Except where specific circumstances make a particular test method inappropriate, TSCA section 4, section 3 of the Federal Insecticide, Fungicide, and Rodenticide Act, as amended (FIFRA), IRLG, or OECD agreed-upon methods will be regarded as suitable for new chemical evaluation purposes. Furthermore, section 5 testing guidance will be periodically revised and updated to reflect these approaches as appropriate.

The tests presented below are grouped into three categories (1) tests for basic chemical and physical properties and other characteristics associated with environmental transport and persistence (Fate Tests), (2) tests for health effects and (3) tests for ecological effects. Each group of tests is preceded by a discussion of the need for information of that type for risk assessment.

The tests and methods set forth below are those which EPA has tentatively identified as appropriate for testing for certain properties and effects. The Agency requests comment on the scientific adequacy of each test and method for assessing the property or effect to which it pertains.

Because there has been considerable confusion concerning EPA's reasons for considering the array of tests presented here, the Agency wishes to emphasize that it does not contemplate recommending that all the tests be performed on any substance. In fact, some of these tests logically could not be performed on the same substance (for example, tests for liquid density and particle size). Similarly, a test for aquatic toxicity would not normally be recommended for a volatile liquid which will not be discharged to water. In fact, as stressed in the discussion in Part I, EPA expects that a manufacturer will design a test plan which is appropriate for his chemical, its characteristics, and use. He will therefore select the tests which are necessary to execute his test plan. The purpose of developing these tests is to establish Agency preferences for particular tests and methods for use in assessing the potential for particular effects. Each recommended test will be available for inclusion in any testing system developed by a manufacturer. EPA will encourage a manufacturer to use recommended tests and methods, but will evaluate on a case by case basis the validity of any other test or method which might be used in premanufacture testing.

The tests and methods presented below vary in their relative degree of validation and standardization. Some have been used by a single laboratory (developed). Others have been thoroughly tested in multilaboratory comparisons (validated) and/or recommended by an appropriate body of experts (standardized). In addition, a few tests have never been performed using the recommended methods; the methods have been constructed from component parts of existing tests.

For each chemical property or effect, the test proposed by EPA is believed to be the most acceptable based on standardization or other stated criteria. In some cases, a conventional or well known test was not selected because of poor cost-effectiveness, inadequate sensitivity, or inappropriateness for assessment. In other cases, there are tests which are believed to be potentially more suitable but which were not selected because they are not yet as well validated as the recommended test. In the latter cases, the Agency will support development and validation to the extent justified by circumstances within the constraints of resource and time limitations. As this development and validation is completed, the tests presented here will be replaced or modified.

The tests are discussed in greater technical detail in a "Draft Technical Support Document" which is being prepared by EPA's Office of Toxic Substances. The "Draft Technical Support Document" will contain a rationale for the choice of each fate and ecological effects test and method and will discuss the extent to which each test has been standardized or validated.

Persons interested in submitting technical commments on the tests and methods described below are urged to first obtain a copy of the "Draft Technical Support Document." (See SUPPLEMENTARY INFORMATION at the beginning of this document.)

Relationship to Other Testing Guidelines and Standards

The development of tests for TSCA section 5 premanufacture testing guidelines relates to other ongoing EPA test development activities. Under section 4 of TSCA and section 3 of FIFRA, EPA is developing proposed testing approaches for a detailed evaluation of selected industrial chemicals and pesticides, respectively. Because the two statutes are similar in purpose, final requirements will, to the maximum extent possible, be identical for those tests common to both.

A. Chemical Fate Tests

A-1. Discussion of Chemical Fate

Assessment of the fate of a chemical substance after its release into the environment is essential for the evaluation of potential risk. Fate, in this sense, is defined as the transport and transformation of a chemical by natural means after it is released to the environment from a point source (e.g., a manufacturing plant or waste treatment plant), a disposal site (e.g., landfill), or a dispersive use. Determination of the fate of a chemical in the environment helps identify:

- The nature of dispersal and ultimate distribution.
- The types of reactions in which the chemical participates during transport.
- The chemical form(s) and concentration(s) to which the environment will be exposed.
- The biological organisms exposed to the chemical.

Two categories of reference tests are recommended to support the preliminary evaluation of the potential fate of a new chemical substance in the environment: transport tests and persistence tests. The first category deals with physical and chemical characterization of substances, including key parameters to allow predictions to be made regarding how and where a substance could be transported after release into the environment. The second category deals with the susceptibility or resistance of a chemical substance to degradation by chemical, photochemical, or biological mechanisms in the environment. Environmental transport and persistence are both important elements in the estimation of potential environmental exposure of a new chemical substance.

The following is a list of chemical and physical properties and environmental fate characteristics that EPA considers potentially important for evaluating a proposed new chemical substance (except where the nature of a particular chemical or its intended use or distribution indicate that such information is unnecessary or unwarranted):

1. Spectral data, including an x-ray diffraction spectrum for inorganic substances, or a mass or alternative spectrum for most other substances.
2. Density, for liquid or solid substances.
3. Solubility in water.
4. Octanol/water partition coefficient.
5. Melting and boiling points.
6. Dissociation constant in water for substances which dissociate in water.

7. Particle size distribution for insoluble solids.

8. Degradation characteristics, noting roughly how fast such degradation occurs upon exposure to oxidants, water, sunlight, microorganisms, and the identity and characteristics of significant degradation products (see test methods in section A4).

9. Flammability, using standard flammability test(s) required by the Department of Transportation for materials in commerce.

10. Vapor Pressure.

11. Adsorption/desorption on particulate surfaces.

Many of the physical/chemical (transport) tests recommended below are reference methods that are already commonly performed by manufacturers to develop sufficient information to move from research to production status or to satisfy requirements of other regulatory agencies such as the Department of Transportation. The most cost-effective, state-of-the-art persistence testing methods were selected whenever possible. For example, biodegradation tests that require radio-labeled substrates were not included in this preliminary set of tests. In some cases, several commonly-used tests are combined into a single recommended protocol because the individual tests could not cost-effectively satisfy the information needs for premanufacture assessment. The Agency, however, has studies under way to validate less expensive experimental methods and will be substituting them as soon as they become available.

Most of the physical and chemical characterization of new chemical substances can be carried out before persistence testing is initiated. This approach provides information for determining the optimum ways to conduct persistence testing and in some cases can serve as a basis for deciding that certain persistence tests are unwarranted. Therefore prudent sequencing of tests can often reduce the time and cost for developing necessary information. This is demonstrated in the following examples:

- Water solubility should be determined early-on since the results can guide the design and conduct of most other tests.
- Boiling point determinations can serve a "range-finding" function for measuring vapor pressure, meaning that only one of the two vapor pressure tests need be conducted.
- An early measurement of hydrolytic degradation should be made, since if a substance hydrolyzes rapidly, tests of adsorption and biodegradation may not be required.
- Soil thin layer chromatography testing could serve as a "range-finding" test for adsorption isotherm tests when needed.

The chemical fate tests and methods presented below were developed to provide sufficient data for an initial evaluation of the fate of a generalized chemical. The choice of test to run for any particular chemical will be governed by the nature of the chemical and its intended use, projected volume, and distribution. Methodologies are evolving and these tests will be replaced or modified as new methods are developed or old ones improved. These tests are state-of-the-art or widely accepted. In either case, they were chosen to yield risk assessment data in the most cost-effective way.

A-2. GENERAL PROVISIONS

A-2.1 *Quality Assurance.* EPA is currently drafting Good Laboratory Practices (GCP) under section 4(b) of TSCA. When these become available, they will provide detailed guidance on proper procedures for laboratory management and the conduct of tests.

A-2.2 *General References.* The testing guidelines proposed under Part 162, Chapter I, Title 40 of the Code of Federal Regulations (Pesticide Guidelines, Subpart D, Chemistry Requirements, Federal Register 43(132): 29708-29724) are recommended as the reference for any chemical fate tests not addressed in the following discussions. As test standards are developed and published under section 4(b) of TSCA and by the Interagency Regulatory Liaison Group (IRLG), they will also serve as tests for use under section 5 of TSCA. Other suggested references are noted under the individual tests contained in these guidelines.

A-2.3 *Test Substance.* (a) Data obtained by tests conducted with the technical grade product to be marketed by the manufacturer will be most relevant for assessment purposes unless that is not the most probable formulation to which the environment will be exposed or unless indicated differently in a specific test method listed below. If the technical grade product is not tested, an explanation of the reason for the substitution would help validate the data for assessment. If possible, a description of the differences between the substance that was used and the market product, including a description of its physical state and chemical nature and how these may affect biological characteristics should be given.

(b) Test results will be more consistent if the lot of the substance tested is the same for the duration of the study, and if the sample is stored under conditions that maintain its purity and stability. If a test substance will not be stable for the duration of the study or, for other reasons, it is not possible to use the same lot throughout the test, subsequent lots of the test substance should be selected that are as nearly identical to the original lot as practicable. Chemical assays should be performed to assure this composition.

(c) Data are more easily and accurately interpreted when the composition of each lot of the test substance, including the name and quantities of contaminants and impurities, is known. An analysis of composition should include the amount of any unknowns, so that 100% ±5% of a sample is accounted for. (Ideally, the amount of any unknown should not exceed 0.5% of the total).

A-2.4 *Data: Suggested Format.* (a) *General.* The data generated by testing are most useful for assessment if submitted in a report format. Such a report should include all information necessary for a generally complete evaluation of the test procedures and results, including information on the facility performing the study, information on the test substance, a description of the test procedures, presentation of the data generated, and a summary and analysis of the test results. The following information is considered valuable for assessment:

(b) *Information on Testing Laboratory/Sponsor.* (1) Name(s) and address(es) of the facility performing the study, and of the sponsor if different from the testing facility.

(2) Laboratory study number

(c) *Information on Test Substance.* (1) Identification of the test substance, including chemical name, chemical abstract number, sponsor code number, synonyms, and molecular structure.

(2) A qualitative and quantitative determination of chemical composition (including names and quantities of known contaminants and impurities, so far as is technically feasible).

(3) Name of the manufacturer, lot number(s) of test substance, method of synthesis, date(s) when received and storage procedures.

(4) Relevant physical properties of the test substance, such as physical state, ph, stability, melting point, purity and solubility.

(5) Identification and composition of any vehicles or other materials used in testing the substance.

(d) *Description of the test procedures.* (1) Specification of test methods, including a full description of the experimental design and procedures, reference(s) to the chosen test method, the length of the study, and the dates on which the study began and ended.

(2) Methods of all chemical analyses performed for determining concentrations of test substances, impurities or degradation products, along with the

precision and accuracy of the test methods.

(3) Appropriate statistical methods used to summarize experimental data, express trends, and evaluate the significance of differences in data from individual test groups. For example, data averages or means accompanied by standard deviation allow assessment of the variability in the raw data. In addition, the standard errors of the means may also be calculated for their use in comparing means from different tests groups. However, notations of statistically significant differences along with the confidence level of probability, could substitute for standard errors.

(4) References to statistical methods for analyzing the data, and to published literature used in developing the test protocal, performing the test, making and interpreting observations, and compiling and evaluating the results.

(3) *Presentation of Data Generated.* All data which would be useful for assessment from each of the test methods.

(f) *Summary Analysis of Test Results.* A summary and analysis of the data, and a statement of the conclusions drawn from the analysis. The summary could highlight positive data or observations and deviations from control data.

A-2.5 *Miscellaneous Considerations.* (a) Since photochemical effects may influence test results, reaction mixtures should be shielded from incident light whenever practical during the experimental period.

(b) The use of trade names in the text is not intended as an endorsement by EPA but is merely intended to illustrate examples of equipment, materials and other facilities suitable for the tests described.

A-3. PHYSICAL/CHEMICAL PROPERTIES, MEASUREMENTS, AND TESTS

A-3.1 *General.* Chemicals discharged into the environment are generally released into one or more of three media: air, water and soil. [1] Discharged materials often do not reside long in one medium but are transported to other media by processes that depend upon the characteristics of chemical substances, as well as meteorological conditions, the flow of surface waters, the location and flow of groundwaters, tidal action, topographic characteristics and the physical and chemical characteristics of soils, sediments, and particulates. An analysis of the transport and fate of discharged chemicals is further complicated because the media can be divided into a variety of subtypes. Thus, water can be fresh, saline, or estuarine, surface or ground, free or bound. Similarly, soils vary widely in structure, compositions, and chemical properties.

The permutations that stem from these variables are enormous. No simple test scheme can predict absolutely how long a chemical will remain in one particular location, or by what mechanism and at what rate it will migrate. The most any test sequence can accomplish is to provide data which a skilled evaluator can integrate with other information to draw presumptive conclusions. These conclusions indicate where a particular chemical is *likely* to be distributed and a rough order-of-magnitude estimate of its concentration. Often the results of such an analysis can rule out the possibility of a chemical entering certain environments in significant concentration. Such conclusions are valuable since they markedly reduce the time, effort, and cost of subsequent testing.

A-3.2 *Water Solubility.* (a) *Objectives.* The water solubility of a chemical is an important characteristic for establishing that chemical's potential environmental movement and distribution. In general, highly-soluble chemicals are more likely to be distributed by the hydrologic cycle than poorly-soluble chemicals.

Water solubility can also affect adsorption and desorption on soils and volatility from aquatic systems. Substances which are more soluble are more likely to desorb from soils and less likely to volatilize from water. Water solubility can also affect possible transformation by hydrolysis, photolysis, oxidation, reduction, and biodegradation in water. Finally, the design of most chemical tests and many ecological and health tests requires precise knowledge of the water solubility of chemicals.

Water solubility is an important parameter for assessment of all solid or liquid chemicals. Water solubility is usually not useful for gases because their solubility in water is measured when the gas above the water is at a partial pressure of one atmosphere. Thus, solubility of gases does not generally apply to environmental assessment because the actual partial pressure of a gas in the environment is extremely low.

(b) *Rationale.* Several methods have been published for determining the saturated water solubility of chemicals. In selecting the method to use, three factors should be kept in mind when determining the *true* saturated equilibrium water solubility. First, most methods define a specific time period for immersion in a constant-temperature bath, after which it is assumed that saturation has been achieved. This is not always accurate, particularly in the case of very hydrophobic chemicals. Second, very hydrophobic solids inevitably form colloids when mixed with water. Some methods do not provide for the removal of these colloidal suspensions and result in inaccurate measurement. Third, water purity is extremely important for meaningful solubility determinations. These factors are important and were all considered in choosing the tests for water solubility recommended below.

The composition of natural waters can vary greatly. Environmental variables such as pH, water hardness, cations, anions, naturally occurring organic substances (e.g., humic and fulvic acids, hemicelluloses) and organic pollutants all affect the solubility of chemicals in water. Some bodies of water contain enough organic and inorganic impurities to significantly alter the solubility of poorly soluble chemicals. Since it is difficult, if not impossible, to define a standard natural water, very pure water should be used instead of natural water in determining the water solubility of chemicals.

When attempting to determine the true saturated equilibrium of very hydrophobic chemicals, it should be recognized that colloids can form and do not readily aggregate. Therefore, it is suggested that the sample be ultracentrifuged at two or three different G values for one half hour at constant temperature until concentration changes are small. This will remove colloidal particles (Biggar and Riggs, 1974).

A recently published method used coupled column liquid chromatography to determine the aqueous solubility of some very hydrophobic organic chemicals (polycyclic aromatic hydrocarbons) (May, et al., 1978). The method seems promising but needs more experimental work on a variety of very hydrophobic chemicals before it qualifies as state-of-the-art. No single method covers the entire range of solubilities in water. Thus, several methods are given to cover the range of water solubilities from the part-per-billion ($\mu g/l$) range to greater than approximately 500 mg/l.

(c) *Methods*—(1) *Test details.* (A) The following considerations should be taken into account when determining the solubility of chemicals in water:

(i) See Mader and Grady (1970) for a discussion of methods for determining the solubility of solutes in liquids before making measurements.

(ii) One should conduct solubility experiments in a thermostatic bath with a shaker, good temperature control, and an accurate temperature measuring device. Determine the solubility at 10, 20, and 30 ± 1° C.

(iii) When dissolving very hydrophobic chemicals in water, as little agita-

[1] Including atmospheric particulates and aquatic sediments.

tion as possible should be used to minimize agglomeration of the finely dispersed particles.

(iv) In some cases, very hydrophobic chemicals tend to adsorb to the surface of the vessels. To minimize this, all transfer vessels should be prerinsed with waterphase aliquots.

(v) The solubility of chemicals which reversibly ionize or protonate (e.g., inorganic acids and bases, carboxylic acids, phenols, and anilines) should be measured at pH 5.0, 7.0, and 9.0. Prepare buffer solutions according to the *Handbook of Chemistry and Physics* (1974–1975).

(vi) Very pure water (e.g., water comparable to reagent grade water from a millipore Milli-Q-System) should be used.

(B) The principal method for determining the solubility of a chemical at saturation is to analyze the solution by some suitable physical or chemical method. Whenever possible, an analytical procedure should be used having a precision within ±5%.

(C) Solubility measurements should be made on analytically pure chemical (greater than 99% by weight pure), if possible. If the chemical contains some impurities, the nature and amount (%) of each impurity should be specified.

(D) *To determine the solubility of solids and liquids in water at concentrations greater than approximately 500 mg/l.* Mader and Grady (1970) described a method to determine the solubility of solids in liquids. The apparatus designed by Lombardo (1967) should be used, with very pure water as the solvent. Equilibrium is approached by undersaturation and oversaturation. When duplicate determinations with both approaches yield concentrations within ±5%, the true saturated equilibrium solubility has been reached.

In addition, Mader and Grady (1970) described a method to determine the solubility of liquids in liquids. Very pure water should be used as the solvent. Equilibrium is approached by undersaturation and oversaturation. When duplicate determinations with both approaches give concentrations within ±5%, the true saturated equilibrium solubility has been reached.

If the concentration of the solute exceeds 10 gm/l, the density of the solution can be determined by weighing known volumes of the solution at the same temperature as the constant-temperature bath. Duplicate determinations should agree to within ±5%. If the concentration of the solute is less than 10 gm/l, the density of the solution does not have to be measured and can be assumed to be the same as the density of water at the temperature of the constant-temperature bath. See the *Handbook of Chemistry and Physics* (1974–1975) for the density of water as a function of temperature.

(E) *To determine the solubility of solids in water from 1 mg/l to approximately 500 mg/l.* The Pesticide Guidelines described a nephelometric method to determine the solubility of solids in water from one to a few hundred ppm (EPA, 1975). For further details on carrying out nephelometric (turbidity) measurements see *Methods of Chemical Analysis of Water and Wastes* (EPA, 1974). Special sonicators should be used to form stable suspensions (Riggs and Biggar, 1965). Very pure water should be used and the experiments conducted in duplicate.

(F) *To determine the solubility of very hydrophobic solids in water.* For very hydrophobic organic chemicals which form colloids, the following method can be used. The chemical should be dissolved in a suitable volatile organic solvent and coated on the walls of a vessel. One should avoid coating the bottom of the vessel. A teflon-coated magnetic stirrer should be carefully placed in the bottom, very pure water added, and the mixture slowly stirred. Concentration-time studies should be conducted to make sure that true saturated equilibrium solubility has been reached. When the concentration reaches a plateau, this indicates true saturated equilibrium solubility. When determining the concentration of chemical in the aqueous phase, the suspension should be centrifuged at two or three different G values for one-half hour at constant temperature until concentration changes are small (Biggar and Riggs, 1974). For several pesticides, centrifugation at approximately 39,000 G removed the colloidal particles corresponding to 0.01 μ particle size (private communication with Biggar). Centrifuge tubes which can be tightly sealed should be used. Solubility experiments should be conducted in duplicate.

(G) *To determine the solubility of hydrophobic liquids in water.* For hydrophobic liquids, the method of McAuliffe (1966) is recommended, with the following modifications: (1) Very pure water should be used; (2) Concentration-time studies should be conducted to make certain that true saturated equilibrium solubility is reached. When the concentration reaches a plateau, this indicates true saturated equilibrium solubility. To make sure that no emulsions persist, the aqueous phase should be checked with a Hach 2100 Turbidimeter (or an equivalent instrument). If the emulsions persist for a long time, high speed centrifugation in tightly sealed tubes is suggested to break the emulsion. Solubility determinations should be conducted in duplicate.

(2) *Calculations and Information Which Should be Recorded.* The method used for determining the saturated water solubility should be given. In describing the analytical method used, an outline of the detailed procedure and the calibration data are considered important, as are the mean solubility and the standard deviation. In addition, if extraction methods were used to separate the solute from the aqueous phase, a description of the method of extraction and recovery data should be provided.

For the nephelometric method, a plot of the turbidity vs. concentration should give a straight line. The best equation for the straight line should be calculated by linear regression analysis and the solubility determined from this equation at the point of zero turbidity. The slope, intercept, correlation coefficient (R^2), and the solubility of each set of experiments should be recorded. The mean solubility and standard deviation should be recorded.

(d) *References.*

Biggar, J.W. and R.L. Riggs. 1974. Apparent solubility of organochlorine insecticides in water at various temperatures. *Hilgardia* 42:383.

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McAuliffe, C. 1966. Solubility in water of paraffin, cycloparaffin, olefin, acetylene, cycloolefin, and aromatic hydrocarbons. *J. Phys. Chem.* 70:1267.

Riggs, R.L., and J. W. Biggar. 1965. Ultrasonic suspension of chlorinated pesticides in aqueous media. *Soil Sci. Soc. of Amer. Proc.* 29:679.

A-3.3 *Octanol/Water Partition Coefficient.* (a) *Objectives.* When a chemical enters a living organism, its accumulation and transport are governed by polarity, water solubility, affinity for fatty tissues, and the nature of potential binding to biological receptors.

The octanol/water partition coefficient is an indicator of bioconcentration potential in fatty tissues in aquatic and other living organisms. The partition coefficient is one factor in determining whether to conduct fish bio-

concentration studies. Its relationship to molecular structure and in some cases toxicity also makes it useful in the assessment of all organic chemicals.

(b) *Rationale.* The octanol/water partition coefficient, P, the ratio of the equilibrium molar concentrations of the chemical in octanol and water, was introduced by Hansch and coworkers to correlate biological activity and chemical structure (Hansch, 1969; Hansch and Fujita, 1964). Hansch and coworkers published many papers on the conventional determination of, and correlations involving, P. The octanol/water partition coefficient is related to the bioconcentration potential of organic chemicals in fish (Neely, et al., 1974). Based on Neely's publication, the partition coefficient has been widely used as a measure of bioconcentration potential in fatty tissues in aquatic and other living organisms.

There are published alternatives to the conventional method of determining the octanol/water partition coefficient used by Chiou, et al. (1977), Hansch, et al. (1967) and Leo, et al. (1971). For example, more rapid methods based on high-pressure liquid chromatography have been developed (Mirrless, et al., 1976; Yamana, et al., 1977; Carlson, et al., 1975; Veith, et al., 1978). In addition, a correlation between the partition coefficient P and the water solubility of some chemicals has been demonstrated (Chiou, et al., 1977). However, these methods are based on a limited number of experiments and none qualifies as state-of-the-art.

The published literature concerning the conventional method for determining the octanol/water partition coefficient indicates that numerous factors, which are discussed in the following section, should be considered. The reasons for choosing very pure water to determine P are discussed in Section A-3.2(b).

(c) *Test Description.* The octanol/water partition coefficient is defined as the ratio of the equilibrium molar concentrations of chemical in octanol and water. It is determined by simultaneously exposing a chemical substance to water and octanol and then measuring the amount of chemical in each phase after equilibration.

(d) *Methods*—(1)*Test Details.* The following brief discussions and test procedures can be used as guidelines to determine the partition coefficient of organic chemicals.

(A) The partition coefficient P, derived using thermodynamic theory (Glasstone, 1946), indicates that in dilute solution

$$P = \frac{C_{octanol}}{C_{H_2O}} \quad \ldots \quad (1)$$

That is, the solute (organic chemical) distributes between octanol and water in such a way that at equilibrium the ratio of the molar concentrations of the solute in the two layers is a constant at a given temperature. Because equation 1 is applicable only in dilute solution, all partition coefficients should be determined at a concentration $C<0.01M$. Since most physical properties of chemicals are reported at 25°C, P should be determined at this temperature. P is an equilibrium constant and can vary to some extent with temperature. Hence, the temperature should be controlled to ± 1°C.

(B) Equation 1 applies only to individual molecular species in solution. If a molecule associates or dissociates in octanol and water, then equation 1 needs to be modified to take into account such speciation changes as ionization, aggregation, and hydration, (Leo, et al., 1971; Glasstone, 1946).

(C) The purity of the solute can affect solubility in octanol and water and may affect the determination of its concentration. Thus, in determining the partition coefficient of a solute, analytically pure material (greater than 99% pure by weight), should be used, if possible. If the chemical contains some impurities, the nature and amount (%) of each impurity should be noted.

(D) Very pure water (e.g., water comparable to reagent grade water from a Millipore Milli-Q-system) should be used. Pure n-octanol (e.g., n-octanol comparable to Eastman Kodak White Label, minimum 98% pure) should be washed sequentially with 0.1N H_2SO_4, with 0.1N NaOH, then with very pure water until neutral. The n-octanol should be dried with calcium chloride and distilled twice in a good distillation column under reduced pressure. This should produce 99.9% pure n-octanol.

(E) In order to insure that equilibrium has been established, gently shake the two-phase system for one hour.

(F) The ratio of the volumes of the two solvents should be adjusted as appropriate for the relative solubility of the solute in octanol and water. By adjusting the volumes, concentration errors are minimized and errors resulting from dividing large numbers by small numbers are kept to a minimum (Hansch and Anderson, 1967; Leo, et al., 1971). Leo, et al. (1971) give an example to illustrate this point.

(G) In determining the P value for any given solute, the octanol and water phases should be analyzed for the solute. Whenever possible, an analytical procedure with a precision within ±5% should be used.

(H) When measuring the partition coefficient of chemicals which reversibly ionize or protonate (e.g., carboxylic acids, phenols, or anilines) pure water at pH 5, 7, and 9 should be used with buffers prepared according to *Handbook of Chemistry and Physics* (1974–1975).

(I) Gentle shaking should be used to minimize the formation of emulsions. Since centrifuging is necessary to separate troublesome emulsions, equilibration should be conducted in ground-glass stoppered centrifuge tubes. Volatile compounds are thus easily handled and a transfer step is eliminated. For volatile compounds, enough solvent should be used so tubes are almost full. In this way, partitioning with air can be neglected (Hansch and Anderson, 1967). Centrifuging at 37,000G at 25°C for 20 minutes should be performed (Chiou, et al., 1977).

(J) Very hydrophobic chemicals (with P in the order of 10^6) require relatively large volumes of the aqueous phase. These solutions should be equilibrated in a large ground-glass stoppered flask, and for the final phase separation transferred to centrifuge tubes that are prerinsed with some of the aqueous phase. After centrifugation, aliquots should be withdrawn from each tube (the transfer vessel should be prerinsed with the water phase) and recombined for analysis.

The methods outlined by Leo, et al. (1971), Hansch, et al. (1967), and Chiou, et al. (1977) are useful as general guidelines to the determination of the partition coefficient of organic liquids and solids. For determination of the partition coefficient of gases, the method of Hansch, et al. (1975) is recommended. The partition coefficient should be determined in duplicate at concentrations $C<0.01M$ and $C_1=0.1C$.

(2) *Calculations and Information Which Should be Recorded.* (A) A description of the procedure of analysis, calibration data and the method used to separate and sample the water and n-octanol phases are considered important. In addition, if extraction methods were used to separate the solute from the n-octanol and the aqueous phases, a description of the extraction method and recovery data should be provided.

(B) Calculations of the partition coefficient using equation 1 for each separate determination at concentrations C and C_1 should be made. The mean value of P and the standard deviation for each concentration C and C_1 should be recorded. If P is not constant at C and C_1, association and dissociation should be considered in determining the true value of P (Leo, et al., 1971; Glasstone, 1946).

(e) *References.*

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A-3.4 *Vapor Pressure.* (a) *Objectives.* Volatilization, the evaporative loss of a chemical, depends upon the vapor pressure of the chemical and on environmental conditions which influence diffusion from the evaporative surface. Volatilization is an important source of material for airborne transport and may lead to the distribution of a chemical over wide areas and into bodies of water (e.g. in rainfall), far from the site of release. Vapor pressure values provide indications of the tendency of pure substances to vaporize in an unperturbed situation, and thus provide a method for ranking the relative volatilities of chemicals. Vapor pressure data combined with solubility data permit calculations of rates of evaporation of dissolved organics from water using Henry's Law constants, as discussed by MacKay and Leinonen (1975) and Dilling (1977).

Chemicals with relatively low vapor pressures, high adsorptivity onto solids, or high solubility in water are less likely to vaporize and become airborne than chemicals with high vapor pressures or with less affinity for solution in water or adsorption to solids and sediments. In addition, chemicals that are likely to be gases at ambient temperatures and which have low water solubility and low adsorptive tendencies are less likely to transport and persist in soils and water. Such chemicals are less likely to biodegrade or hydrolyze, but are prime candidates for photolysis and for involvement in adverse atmospheric effects (e.g., smog formation, or stratospheric alterations). On the other hand, nonvolatile chemicals are less frequently involved in significant atmospheric transport, so concerns regarding them should focus on soils and water.

Knowledge of the vapor pressure is important for assessment of all chemicals except those with a standard boiling point of 30°C or less, and those whose vapor pressure at ambient temperatures is less than 10^{-7} torr. Compounds with a low boiling point, if free in the environment, would readily vaporize under ambient conditions, and thus would be classified as highly volatile. For such materials, the boiling point at standard atmospheric pressure would confirm high volatility.

(b) *Rationale.* Since no single vapor pressure procedure applies to the entire range of vapor pressures at ambient temperatures, more than one method is needed to measure vapor pressures from 10^{-7} to 760 torr. A standardized method, ASTM D-2879-70 (ASTM, 1974), is used for pure liquids to determine vapor pressures from 1 to 760 torr. A gas saturation method is proposed to determine vapor pressures of compounds from 10^{-7} to 10 torr. This method is the best technique available to measure low vapor pressures.

Most of the alternative methods for measuring vapor pressure are unsuitable because they cover only a limited vapor pressure range, are complex, are limited to very precise measurements, or apply only to specific chemicals.

Boiling point methods, such as the one using the Ramsey and Young apparatus, were rejected because the accuracy is very poor below 10 torr (Thomson and Douslin, 1971).

Effusion techniques, particularly those employing the Knudsen effusion apparatus, measure vapor pressures from 10^{-7} to 10^{-2} torr and provide some good data (Hamaker and Kerlinger, 1969). These methods were rejected due to the great difficulty of working with a system under vacuum and the need to keep the capsule space saturated with vapor during measurement. The lack of equilibrium saturation may have caused inaccurate published vapor pressure data (Spencer and Cliath, 1970).

The isoteniscope method is a standardized method for determining the vapor pressure of certain liquid hydrocarbons. The procedure is relatively simple, and the sample may be purified within the equipment by removing dissolved and absorbed gases until the measured vapor pressure is constant. These procedures do not remove higher-boiling impurities, decomposition products, or compounds that boil close to, or form azeotropes with, the material being tested.

Gas saturation procedures described by Spencer and Cliath (1969) use relatively simple equipment and yield precise and readily obtained results. The same methods also apply to laboratory environmental simulations. The vapor pressure is computed by assuming that the total pressure of a mixture of gases equals the sum of the pressures of the component gases. The partial pressure of the vapor under study may be computed from the total gas volume and the weight of vaporized material. Direct vapor density measurements by gas saturation techniques apply more directly to practical problems related to the volatilization of chemicals than other techniques (Spencer and Cliath, 1970). Where applicable, use this method for determining the vapor pressure of compounds and the effective vapor pressure of compounds in soil or water.

(c) *Test Description.* Vapor pressure is measured by gas saturation or with an isoteniscope. The gas saturation method, which applies from 10^{-7} to 10 torr measures the vapor concentration of the test compound in a flowing, inert gas under equilibrium conditions. The isoteniscope method, which applies to vapor pressures from 1 to 760 torr, involves placing a liquid sample in a thermostated bulb connected with a mercury manometer and a vacuum pump. Enough liquid is evaporated, repeatedly, to drive out all other gases, until further evacuation gives no lowering of the vapor pressure.

(d) *Methods*—(1) *Test Details.* The vapor pressure of compounds can be determined by one of the following procedures:

- The isoteniscope method for vapor pressures of liquids with vapor pressures of 1 to 760 torr. A description of this method appears as ASTM method D-2879-70 (ASTM, 1974).

- The gas saturation method for vapor pressures of 10^{-7} to 10 torr. Spencer and Cliath (1969) gives a description of the apparatus, procedures, and calculation. The amount (in grams) of test material evaporated per unit time should be calculated at each of three or more gas flow rates and the grams vs. flow rate should be plotted. If equilibrium conditions were established, the plot is a straight line. If a straight line is not obtained, the experimental conditions should be adjusted until equilibrium is achieved.

Determinations with the sample should be made in triplicate at each of three temperatures (10°, 20° and 30°C) unless a change of state (solid to liquid or solid to vapor) or a transition point (a change from one crystalline form to another) occurs in that temperature range. If a change of state or transition point occurs in the temperature range 10°C to 30°C, the following information is considered important:

- Nature of change.
- Temperature at which the change occurs at atmospheric pressure.
- Vapor pressure at 10° and 20°C below the temperature at which the transition or change of state is noted, and at 10° and 20°C above that temperature (unless the transition is from solid to gas).

Determine vapor pressure at 10°, 20°, and 30°C for a standard reference material suitable to the method. Calculate the vapor pressure and plot the log (base 10) of the vapor pressure vs. the reciprocal of the temperature in °K. The plot should be a straight line.

(2) *Calculations and Information which should be Recorded.* The average calculated vapor pressure for the test material and the calculated vapor pressure for the reference material at each temperature are considered important, as is a plot of log p vs 1/T for the test material and for the reference material.

A description of the experimental method, including details on difficulties experienced, and any other pertinent information should be provided.

(e) *References.*

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A-3.5 *Adsorption.* (a) *Objectives.* The affinity of a chemical substance for particulate surfaces is an important factor affecting its environmental movement and ultimate fate. Chemicals that adsorb tightly may be less subject to environmental transport in the gaseous phase or in solution. However, chemicals that adsorb tightly to soil particles may accumulate in that compartment. Substances which are not tightly adsorbed can transport through soils, aquatic systems and the atmosphere.

For some substances, results of adsorption testing hold little value in assessment. If, for example, a substance is a water insoluble gas at atmospheric preasure and ambient temperature, other chemical fate properties such as volatility might far outweigh the effects of adsorption into soil or sediment particles in determining environmental fate.

(b) *Rational.* A number of laboratory tests consider the effects of soil and sediment adsorption on mobility. The two tests recommended for use are the adsorption isotherm (soils and sediments) and soil thin layer chromatography (soils only). These tests were selected for their relatively low cost, uncomplicated test procedures, wide usage and acceptance, and low labor requirements. Both tests are recommended for use by the Pesticide Guidelines (EPA, 1978).

The use of soil columns was considered but was not recommended because of the following problems: (a) the difficulty of standardizing column packing, (b) the large amounts of soil and chemical required, and (c) the excessive time and labor requirements.

The octanol/water partition coefficient was not considered as a preliminary test for adsorption because the colloid and soil chemistry literature does not conclusively indicate its applicability for predicting movement and persistence of a wide range of chemicals for a wide spectrum of soils and sediments.

Efforts to identify a method for measuring the adsorption of chemicals to atmospherically-transported particles are being made. However, no method is presently recommended.

(c) *Test Description.* Soil, the unconsolidated mineral material on the immediate surface of the earth, and sediment, the unconsolidated material deposited at the bottom of natural waters, are used as adsorbents in these tests. Two test methods, the adsorption isotherm and thin layer chromatography (TLC), are recommended to predict chemical mobility as a function of adsorption on particulate surfaces. Either the adsorption isotherm or the soil TLC method can be used to determine the test chemical's affinity for soil, but only the adsorption isotherm method is suitable for determining the test chemical's affinity for sediment.

A mobility class is assigned to the test compound based upon the compound's interaction with the adsorbents. For preliminary tests, mobility classes are assigned using a limited number of adsorbents. Therefore, extrapolation to a wider spectrum of absorbents which the test chemical may ultimately encounter in the environment cannot be made without further testing.

An adsorption isotherm is a partition diagram which describes the distribution of the test chemical between a solid absorbent and the solution in equilibrium with it. The "batch" technique is the typical method for determining and adsorption isotherm. A series of solutions containing various concentrations of the chemical are equilibrated with the adsorbent. Either the amount of chemical initially added or the amount of chemical adsorbed by the adsorbent is plotted as a function of the equilibrium solution concentration of the chemical. The resulting curve or plot is the adsorption isotherm.

Soil TLC has a fundamental analogy in conventional chromatography. Movement in soil TLC is usually measured as the frontal R_f of a spot or streak (the test chemical) which results from water movement up a thin layer chromatography plate using soil as the adsorbent. This method should be used only with test chemicals that can be analytically determined on the TLC plate in the presence of soil colloids and soluble soil organic components.

(d) *Methods*—(1) *Test Details*—(A) *Adsorption Isotherm.* A typical method of conducting an isotherm determination is described in Weber (1971). The Pesticide Guidelines (EPA, 1978) also list references to procedures for adsorption studies (Aharonson and Kafkafi, 1975; Harvey, 1974; Murray, et al., 1975; Saltzman, et al., 1972; Wu, et al. 1975). This protocol is based upon these references, with certain modifications to minimize the disparity in materials, laboratory methodology, data presentation and data interpretation.

(i) One sediment and one soil should be used in this study. The soil selected for this study should have a pH between 4 and 8, an organic matter content between 1 and 8%, a cation exchange capacity greater than 7 MEQ/100 GM, and a sand composition of less than 70%.

(ii) Duplicate samples with a 1:5 adsorbent:solution ratio should be used (for example, a 10 ml solution with 2 gms of oven-dry soil (dry at 90°C for 24 hours)). Soils should be sieved with a 100 mesh stainless steel or brass screen before testing. Sediments should be air dried.

(iii) Distilled-deionized H_2O, adjusted to pH 7 by boiling to remove CO_2, should be used as the solvent.

(iv) Containers composed of materials that adsorb negligible amounts of

the test chemical should be used. Other details of container design are not critical, except that the liners of screw caps should be teflon. The six concentrations listed below should be added to the containers and equilibrated without sediment or soil for 48 hours. The amount of adsorption by container walls should be measured and recorded.

(v) Six initial concentrations, 0.00, 0.05, 0.25, 1.25, 6.25 and 31.25 micrograms/ml, should be equilibrated with each adsorbent. If low solubility of the test chemical prohibits the use of some of these concentrations, the adsorption isotherm will have a high and a low equilibrium solution concentration at least one order of magnitude apart. The experiment should be run with a minimum of six different equilibrium solution concentrations at 20°C.

(vi) Immediately after addition of the solution the container should be vigorously agitated with a vortex mixer or similar device. The container should be shaken for 5 minutes once every hour for 48 hours at a rate to keep the adsorbent in suspension during the shaking period. After equilibration the suspensions should be centrifuged at a high speed (at least 20,000 g) for 10 minutes. The aqueous equilibrium solution should be stored at 5°C.

(vii) The chemical adsorbed on the colloidal surface should be extracted with an organic solvent in which the test chemical is soluble (minimum 1 gm/liter) and a mass balance performed. A volume of organic solvent equal to the original volume of aqueous solution (used to attain equilibrium) should be added to the adsorbent and the container shaken vigorously for 10 minutes. The mixture should then be centrifuged at a minimum of 20,000 g for 10 minutes. This extraction procedure should be performed three times. The aqueous phase and the organic solvent extracts should be analyzed for the parent chemical.

(viii) The chemical remaining in the aqueous phase and the organic solvent extracts should be characterized and measured by UV absorbance. GC retention time, mass spectrometry or other methods appropriate and specific for the particular chemical.

(B) *Soil Thin Layer Chromatography.* The typical method of conducting a soil thin layer chromatography (TLC) study is described by Helling (1971a, 1971b, 1971c), and Helling and Turner (1968). The following protocol is based upon these references.

(i) One soil with a pH between 4 and 8, an organic matter content between 1 and 8%, a cation exchange capacity greater than 7 meg/100 gm and which is less than 70% sand should be used.

(ii) Soils should be prepared by sieving to remove stones, coarser sand fractions, and large plant fragments. Any crushing or grinding involved should reduce soil aggregate size but should not create more fine particles (silt and clay) than originally present. Soils should be sieved at 250 μm to remove coarse (500–2,000 μm) and medium (250–500 μm) sand fractions. Earlier testing showed that removal of a portion of the sand did not affect mobility of a test compound but did aid in achieving a more uniform soil layer.

(iii) Distilled-deionized H_2O adjusted to pH 7 by boiling to remove CO_2 should be used.

(iv) Water should be added to a sieved soil until a smooth, moderately fluid slurry is attained. Approximately 0.75 ml H_2O added for each gram of soil will provide a moderately fluid slurry. The soil slurry should be applied to clean glass plates quickly to prevent particle size segregation.

(v) The soil slurry should be spread evenly across the plate by (1) use of a variable thickness plate spreader or (2) use of a glass rod. If a glass rod is used, the layer thickness should be controlled by affixing multiple layers of masking tape along the plate edges. Soil layer thickness should be 500–750 micrometers.

(vi) The plates should be air dried for a minimum of 24 hours after slurry application.

(vii) The purest grade of test chemical available should be used in this study. Impurities cannot be distinguished from degradation products and may indicate the need for degradation product identification when in fact, none exists.

(viii) At least three replicate plates for each soil should be used. A horizontal line should be scribed through the soil layer 11.5 cm above the base. The test chemical should be spotted 1.5 cm from the edge and the amount recorded. Approximately the same quantity should be used for each plate. For radio labeled materials, 0.5–5 μg containing .01–.03 μCi of ^{14}C labeled compound is appropriate.

(ix) The plate should be immersed vertically in a closed chromatographic chamber containing 0.5 cm H_2O. The plate should be removed from the chamber when the solvent front has migrated to the 11.5 cm line.

(x) R_f values should be determined by visualization, zonal extraction, plate scanning or other methods suitable for detecting the test chemical and the techniques for R_f measurement should be described.

(2) *Calculations and Information Which Should be Recorded*—(a) *Adsorption Isotherm.* The average value of the adsorbed chemical, x/m, as a function of equilibrium concentration, C_e, should be plotted for each soil according to the equation $(x/m)=KC_e^{1/n}$. The quantity x refers to the amount of adsorbed chemical per unit amount of adsorbent m (usually expressed as μg/g soil). The adsorbed chemical is defined as the difference between the initial concentration of the chemical added to the soil-solution system and the equilibrium solution concentration of chemical. The quantities K and n are constants. The Freundlich adsorption coefficient can be obtained by plotting log (x/m) vs. log C_e, yielding a linear slope of 1/n. The Freundlich constant K and the constant 1/n should be recorded along with all raw data (see Table 2).

A mass balance for the parent compound should be developed by subtracting the combined concentrations of the parent chemical in the aqueous phase and in the organic solvent extracts from the initial concentration of parent compound and recording the difference. A reasonable hypothesis should be submitted to account for any discrepancy greater than 5% between the initial concentration and the concentration found after adsorption, along with any appropriate supportive data.

The following information on the selected soil is considered important: soil order, soil series, classification, sample location, horizon, particle size analysis, percent organic matter, pH, percent carbonate as $CaCO_3$ (for soils with basic pH), cation exchange capacity, extractable cations, and clay fraction mineralogy. The following information on the selected sediment is considered important: location, particle size analysis, percent organic matter, pH, cation exchange capacity and clay fraction mineralogy. Black (1965), Hesse (1971) and Jackson (1969) describe methods of analysis. Soils with widely differing physical and chemical properties, but representative of most of the soil orders in the United States, are listed in Table 1 and are suggested for use in this test.

If reference compounds with known adsorption isotherms are used, record their adsorption isotherms.

(B) *Soil Thin Layer Chromatography.* The following information should be recorded: (i) approximate concentration of the test chemical applied to the plates, (ii) detailed description of the analytical technique used in R_f determination, (iii) the mean frontal R_f value with the standard deviation for each soil tested. (iv) a photograph or diagram of the TLC plate and showing the entire leaching pattern (from 1.5 to 11.5 cm) (see Table 2); and (v) the physical/chemical properties of the soil as described in (d)(1)(B)(i).

(C) *Use of the Data.* The Freundlich constant K and the R_f will help place

the chemical into a generalized mobility class. Table 3 summarizes the general relationship between the soil/solution partition coefficient K, R_f and soil mobility. The chemical will have a mobility class for each soil tested.

(e) *References.*

Aharonson, N., and U. Kafkafi. 1975. Adsorption, mobility and persistence of thiabendazole and methyl 2-benzimidazole carbamate in soils. *J. Agr. Food Chem.* 23:720–724.

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Helling, C. S. 1971b. Pesticide mobility in soils II. Applications of soil thin layer chromatography. *Soil Sci. Soc. Am. Proc.* 35:737–743.

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[6560-01-C]

Table I

Physical and Chemical Properties[1] of Soils
Suggested for Use in Conjunction With Protocol

For Adsorption Tests[2]

SOIL ORDER:	Alfisol	Aridisol
SOIL SERIES:	Crider	-
CLASSIFICATION:	Typic Paleudalf fine-silty, mixed, mesic	Salorthid
LOCATION:	Gallatin County, Ill.	California
HORIZON:	A	
% SAND:	1.2	
% SILT:	86.6	
% CLAY:	12.2	
% ORGANIC MATTER:	1.74	
CARBONATE AS $CaCO_3$:	-	
pH (1:1 H_2O):	7.2	
CATION EXCHANGE CAP. (MEQ/100GMS)	13.5	
EXTRACTABLE CATIONS (MEQ/100GMS)		
Ca	8.4	
Mg	2.8	
Na	-	
K	0.7	
H	1.6	
SPECIAL CHEMICAL/MINERALOGICAL FEATURE:		
CLAY FRACTION MINERALOGY:	>50% Montomorillonite 5-20% Mica <5% Kaolinite (36-120 cm depth)	Saline

SOIL ORDER:	Histosol	Inceptisol
SOIL SERIES:	Rifle	Maile
CLASSIFICATION:	Typic Borohemist	Hydric Drystrandept, Thixotropic, isomestic
LOCATION:	Michigan	Tallamook County, Ore.
HORIZON:		A
% SAND:		15
% SILT:		53.6
% CLAY:		31.4
% ORGANIC MATTER:		15.4
pH (1:1 H_2O):		5.3
CATION EXCHANGE CAP. (MEQ/100GMS)		103.6
EXTRACTABLE CATIONS (MEQ/100GMS)		
Ca		6.7
Mg		1.2
Na		0.4
K		0.4
H		94.9
SPFCIAL CHEMICAL/MINERALOGICAL FEATURE:	Histic Epipedon	Allophane
CLAY FRACTION MINERALOGY:		33-50% Vermiculite 20-33% Kaolinite 5-20% Quartz 34% Allophane (68-125 cm depth)

[1]Those physical and chemical properties not listed will be published when available.

[2]Soils listed here will be distributed by the National Bureau of Standards.

Table I (continued)

SOIL ORDER:	Inceptisol	Mollisol	Mollisol
SOIL SERIES:	Toledo		Walla Walla
CLASSIFICATION:	Mollic Haplaquept fine, illitic, nonacid, mesic	Natriboroll	Typic Haploxeroll coarse-silty, mixed mesic
LOCATION:	Ohio	North Dakota	Salt Lake County, Ut.
HORIZON:			A
% SAND			30.4
% SILT:			46.5
% CLAY:			23.1
% ORGANIC MATTER:			2.99
CARBONATE AS $CaCO_3$:			-
pH (1:1 H_2O):			7.3 (Saturated Paste)
CATION EXCHANGE CAP (MEQ/100GMS)			26.7
EXTRACTABLE CATIONS (MEQ/100GMS)			
Ca			15.4
Mg			5.1
Na			0.2
K			0.9
H			4.0
SPECIAL CHEMICAL/MINERALOGICAL FEATURE:	Illitic Dolomitic		-
CLAY FRACTION MINERALOGY:			33-50% Kaolinite 20-33% Mica 5-21% Montmorillonite (0-41 cm depth)

SOIL ORDER:	Mollisol	Ultisol	Ultisol
SOIL SERIES:	Sharpsburg	Bladen	Malbis
CLASSIFICATION:	Typic Argiudoll fine, montmorillonitic mesic	Typic Albaquult, clayey, mixed, thermic	Plinthic Plaeudult fine-loamy, siliceous, thermic
LOCATION:	Tama County, IA	Liberty County, GA	Johnston County, NC
HORIZON:	A	A	A
% SAND:	2.2	55.6	64.1
% SILT:	69.2	37.5	27.2
% CLAY:	28.6	6.9	8.7
% ORGANIC MATTER:	2.35	2.2	0.89
CARBONATE AS $CaCO_3$	-	-	-
pH (1:1 H_2O):	5.7	4.1	4.9
CATION EXCHANGE CAP. (MEQ/100GMS)	27.2	14.6	7.4
EXTRACTABLE CATIONS (MEQ/100GMS)			
Ca	13.9	1.80	1.40
Mg	3.4	0.90	0.24
Na	-	0.10	0.01
K	0.5	0.10	0.22
H	9.3	11.70	5.50
SPECIAL CHEMICAL/MINERALOGICAL FEATURE:	Montmorillonite	High Aluminum	Vermiculite Chlorite Kaolinite Gibbsite
CLAY FRACTION MINERALOGY:			Vermiculite Chlorite Intergrade 20-33% <5% Vermiculite Kaolinite 35% Gibbsite 12% Quartz 7% (0-51 cm depth)

Table I (continued)

SOIL ORDER:	Vertisol
SOIL SERIES:	Houston Black
CLASSIFICATION:	Udic Pellustert Fine, Montmorillonitic thermic
LOCATION:	Collin County, TX
HORIZON:	A
% SAND:	23.1
% SILT:	40.7
% CLAY:	36.2
% ORGANIC MATTER:	1.05
CARBONATE AS $CaCO_3$:	-
pH (1:1 H_2O):	6.4
CATION EXCHANGE CAP.:	28.4
EXTRACTABLE CATIONS (MEQ/100GMS)	
Ca	23.0
Mg	4.3
Na	0.3
K	0.8
H	4.5
SPECIAL CHEMICAL/MINERALOGICAL FEATURE:	-
CLAY FRACTION MINERALOGY:	Montmorillonite

Table II

Suggested Format For Data Submission For Adsorption Isotherm Protocol

Soil	Sediment
	SAMPLE LOCATION:
SOIL ORDER:	
SOIL SERIES: CLASSIFICATION	
LOCATION:	
HORIZON:	
% SAND:	% SAND:
% SILT:	% SILT:
% CLAY:	% CLAY:
% ORGANIC MATTER:	% ORGANIC MATTER:
CARBONATE AS $CaCO_3$	
pH (1:1 H_2O):	pH:
CATION EXCHANGE CAP. (MEQ/100GMS)	% INSOLUBLE CARBONATES:
EXTRACTABLE CATIONS (MEQ/100GMS)	CATION EXCHANGE CAPACITY:
Ca	
Mg	
Na	
K	
H	
SPECIAL CHEMICAL/MINERALOGICAL FEATURE:	CLAY FRACTION MINERALOGY:
CLAY FRACTION MINERALOGY:	

Table II (continued) Soil TLC Data Format

SOIL:

AMOUNT OF CHEMICAL APPLIED:

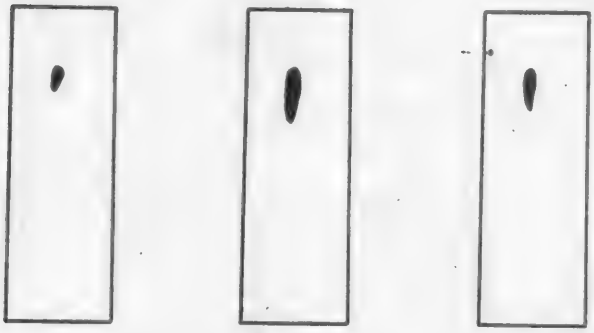


PLATE 1
R_F=

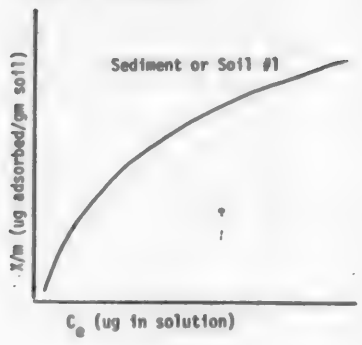
PLATE 2
R_F=

PLATE 3
R_F=

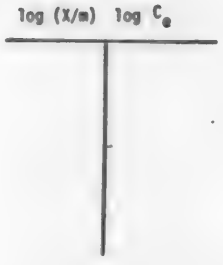
Mean R_F:

Standard Deviation:

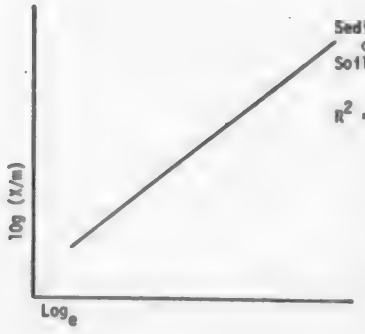
Table II (continued)



Adsorption Isotherm Data Format



K=
1/n=



Mass Balance

initial ug/gm soil added	ug/gm not accounted for	% not accounted for.

TABLE III.—*The General Relationship Between the Soil/Solution Partition Coefficient K, R_f and Soil Mobility*

K_{oc}	R_f	Mobility class	Distance surface applied chemical may leach [1]
0.1	0.95	Very mobile	
1	0.80		Much of chemical leaches through top 20 cm soil into subsoil.
10	0.25	Mobile	Much of chemical leached into soil, but peak concentration in top 20 cm soil.
10^2	0.10	Low mobility	Only small amount of leaching and peak concentration normally in top 5 cm soil.
$10^{2.5}$	0.00		
10^3	0.00		
10^4	0.00	Immobile	No significant leaching.

[1] If rain exceeds evapotranspiration by 10 inches assuming no degradation.

[6560-01-M]

A-3.6 *Other Physical/Chemical Parameters*

A-3.61 *Boiling / Melting / Sublimation Points.* (a) *Objectives.*—The boiling point of a liquid is the temperature at which its vapor pressure equals the pressure of its surrounding environment. The melting point (and/or freezing point) of a chemical is the temperature at which the solid and liquid forms of the chemical are in equilibrium. The sublimation point is the temperature below the melting point at which the vapor pressure of the solid equals atmospheric pressure.

These measurements could provide the information necessary to determine the likely physical state of a chemical at ambient temperatures. A boiling point near ambient temperatures would indicate the likelihood of significant exposure via inhalation to persons in close proximity to the uncontained chemical, and such data may indicate the extent of environmental release through vaporization. Sublimation point determinations could provide a quality assurance check on vapor pressure determinations.

The lowest temperatures commonly occurring in the winter in the United States are approximately minus 30°C. Many compounds that boil between 150 and 200°C have significant vapor pressures ($>10^{-1}$ Torr) at ambient temperatures. Therefore, the boiling point should be determined on compounds that boil between minus 30° and +200° at atmospheric pressure.

(b) *Rationale.* Several methods are offered as examples for boiling/melting/sublimation point determinations. The exact method used is not very important, but it should be properly conducted. The single most important consideration in most of these methods is the use of a slow rate of heating. The following tests are the only methods that were considered; none were rejected.

(c) *Methods*—(1) *Test details.* Some examples of procedures that may be used are those found in ASTM E324, D1078-70, D3451, CIPAC MT 2, the vapor pressure section of this package (A-3.4). For differential thermal analysis/scanning calorimetry (DTA/DSC) methods, ASTM E487, E472, and E537 should be used. Measurements of % weight loss as a function of time at different constant temperatures (thermogravimetry) could provide useful sublimation data. Vapor pressure data and DTA-DSC data are only relevant if phase changes have been noted.

(2) *Calculations and Information Which Should be Recorded.* For vapor pressure determinations, the data as described in the vapor pressure section of this package (A-3.4) should be submitted. For DTA/DSC determinations, a very brief description of the method used should be submitted along with the pertinent data obtained. If an ASTM or CIPAC method is used, the method should be cited and the pertinent data included.

(d) *References.*

ASTM. *1974 Annual ASTM Standards.* Philadelphia, PA.

CIPAC Handbook. 1970. *Analysis of Technical and Formulated Pesticides.* Vol. I. Compiled by R. De B. Ashworth, et al. Edited by G. R. Raw. CIPAC, Ltd., Hertfordshire, England, Chapter 7.

A-3.62 *Density/Specific Gravity*—(a) *Objectives.* Density is the mass per unit volume of a chemical substance at a specified temperature. Specific gravity is the ratio of the mass of a given volume of a substance at a specified temperature to the mass of the same volume of water at a specified temperature.

Measurements of the density of solids and the specific gravity of liquids indicates whether immiscible, low-reactivity chemicals will sink or float in a body of water. The density of gases indicates whether the gas will be transported along the ground, possibly subjecting surrounding populations to high exposure, or whether it will disperse rapidly.

(b) *Rationale.* The choice of method is not critical, but should be suitable for the chemical. The accuracy required for density data in risk assessments is approximately ± 0.1 g/cm³. Accuracy is especially critical for numbers near the density of water (1.0 g/cm³ at 20°C). The air (or helium) comparison pycnometer method is the only method suggested that has not been standardized by ASTM or CIPAC. The method is easy, reproducible, quick, and accurate. The density of gases will be calculated by EPA staff assuming ideal gas behavior unless actual gas density measurements are made.

The only methods considered were those listed below.

(c) *Methods.*—(1) *Test details.* The density of solids should be determined at 20 ± 3°C, by one of the following methods: CIPAC MT3 (for solids) or ASTM (35) D792-66.

The specific gravity for liquids should be determined at 20 ± 3°C relative to water at 20°C. The following methods are suitable: CIPAC MT3 (for liquids), ASTM (23) D941-55, (23) D1480-62 or (23) D1481-62.

In addition, the air (helium) comparison pycnometer can be used at 20 ± 3°C, following manufacturer's recommendations.

(2) *Calculations and Information Which Should be Recorded.* The method used should be very briefly described. If an ASTM or CIPAC method was used, merely cite the method. Density data should be recorded in units of g/cm³ and the specific gravity reported relative to water at 20°C.

(d) *References.*

ASTM. *1974 Annual ASTM Standards.* Philadelphia, PA.

CIPAC Handbook. 1970. *Analysis of Technical and Formulated Pesticides.* Vol. I. Compiled by R. De B. Ashworth, et al. Edited by G. R. Raw. CIPAC, Ltd., Hertfordsnire, England, Chapter 7.

A-3.63 *Dissociation Constant*—(a) *Objectives.* The dissociation constant is an equilibrium constant and is a measurement of the extent to which an ionizable substance breaks up into charged constituents, usually because of the effect of solvent on the dissolved chemical.

The dissociation constant of a chemical can be used in assessing the aquatic, terrestrial, and metabolic fate of the chemical. Dissociated species may precipitate in water depending on certain factors, such as water hardness. The ionic charge of a compound also affects its ability to bind to certain soils and sediments. In addition, metabolism and partitioning (between lipid or octanol and water) of chemicals is affected by the extent of dissociation.

Solids and liquids that have structures that indicate a pK value between three and eleven should be tested. This includes pKa, pKb, and other "first order" solvolysis reactions. Pure samples (99.9+%) should be used, or the nature of impurities and their effect on pK value should be assessed. Compounds with water solubilities below that necessary for the method

discussed (below 10^{-3} M) need not be tested.

(b) *Rationale.* The three methods cited are potentiometric titration, spectrometry (relative absorbances), and conductivity. Each is a classic, well established method, and were the only methods considered. These methods can be used at solubilities from 10^{-3} to 0.5 M, are reproducible and can be performed by most competent chemical laboratory technicians.

(c) *Methods*—(1) *Test details.* The dissociation constant of Brönsted acids and bases should be measured by potentiometry (a pH meter titration with strong acid or base) or by the method of relative absorbances from the relationship:

$$pH = pK - \log \frac{[H_xA]}{[(H_{x-1}A)^-]}$$

The dissociation constant should be determined at 25 ± 1°C, to ± 0.1 pK unit.

For salts and organic compounds that contain functional groups that may be solvolyzed and acids and bases of low water solubility, a conductivity cell should be used. Successive dilutions with the concentration of the ionizing species below 0.01 M should be used, since higher concentrations may cause the dissociation "constant" to vary (MacInnes and Shedlovsky, 1932). The temperature should be held at 25 ± 1°C.

(2) *Reference Compounds.* The dissociation constant of one of the following compounds should be measured at the same time and at the same facility as the new chemical. The appropriate reference compound should be selected based on structural and pK similarities with the new chemical.

acetic acid	phosphoric acid (step 2 pKa)
benzoic acid	ammonium hydroxide
o-, m-, or p-nitrophenol	cyclohexylamine

(3) *Calculations and Information Which Should be Recorded.* For pH titrations, a plot of pH vs volume of standard acid or base should be provided, with accompanying data, including the temperature and pH of buffers used to standardize the pH meter.

For spectroscopic methods, the molar absorptivities of the acid and base forms of the chemical, tabulated data of

$$\log \frac{[HA]}{[A^-]}$$

at various pH values, calculations of pK and the type and model number of the spectrometer should be recorded.

For conductivity measurements, the cell constant and the solution conductivity of the instrument, conductance data (in units of μmhos/cm) and calculations of pK should be recorded.

(d) *References.*

For pH Titrations

ASTM. 1975. *Annual ASTM Standards.* Philadelphia, PA. D1293.

APHA/AWWA/WPCF. 1978. *Standard Methods for the Examination of Water and Wastewater.* 14th ed. American Public Health Association, Washington, D.C. Standard Method 424.

Nelson, H.H., and S.D. Faust, 1969. Acidic dissociation constants of selected aquatic herbicides. *Eviron. Sci. Techno.* 3(11): 1186–1188.

For Conductometry

ASTM. 1974. *Annual ASTM Standard.* Philadelphia, PA. D1125.

APHA/AWWA/WPCF. 1976. *Standards Methods for the Examination of Waste and Wastewater.* 14th ed. American Public Health Association, Washington, D.C. Standard Method 205.

Albert, A., and E.P. Serjeant, 1962. *Ionization Constants of Acids and Bases,* John Wiley & Sons, Inc., New York, NY. pp. 93–105.

MacInnes, D.A., and T. Shedlovsky 1932. The ionization constant of acetic acid. *J. Amer. Chem. Soc.* 54:1429.

For Absorbance

Albert & Serjeant, 1962. op cit. pp. 69–92.

A-3.64 *Flammability / Explodability.*—(a) *Objectives.* The tests in this section include flash point, flammable limits for gases, autoignition temperature, thermal explodability, and shock explodability. The flash point is the temperature at which a liquid or volatile solid gives off a vapor sufficient to form an ignitable mixture with the air near the surface of the liquid or within the test vessel (National Fire Protection Association (NFPA), 1969). "Flammable limits for gases" are the maximum and minimum concentrations of gas and air mixtures that will ignite. The autoignition temperature is the temperature at which a substance will spontaneously ignite in the absence of a spark or flame source. Explodability is the capability of a substance to undergo an uncontrolled, fast, violent chemical reaction resulting in a sudden increase in pressure.

Knowledge about the explosion and fire potential of new chemicals is important for assessing the acute risk they may pose to human health. Information from a flash point determination could indicate what controls, if any, are necessary on the chemical's thermal environment, and possible ignition sources. Information from a gas flammability test could indicate what concentrations of gas in air are likely to pose a fire hazard. Autoignition data could be useful in assessing the potential of new chemicals to spontaneously ignite upon contact with a hot surface or because of heating from some other source. The impact explodability affects the risk of handling large quantities of chemical (the force of a falling container is proportional to mass). Thermal explosion data could be a useful parameter for assessing the risk posed by handling or using various quantities of a chemical. The probabilities of thermally-induced explosion occurring are independent of the weight of the chemical present except as this may affect the rate of heating.

Flash points are appropriate for chemicals that are liquid below 60°C. Gas flammability is appropriate for chemicals that are gaseous under conditions of intended use, unless the chemical has a totally oxidized structure (e.g., BF_3, CO_2). Autoignition is appropriate for chemicals that come into direct contact with hot surfaces (100°C or more). Shock and thermal explodability testing is potentially applicable to all solid and liquid chemicals. (The intended use of a new chemical will be especially important in determining applicability for these tests).

(b) *Rationale.* The flash point methods cited are all closed cup methods. Open cup methods generally give higher results, and reaction conditions are not as well controlled. Also regulations of the Department of Transportation (DOT), Department of Labor (OSHA) and EPA (Office of Pesticide Programs and Office of Solid Waste) all require closed cup methods. However, it has been suggested that open cup methods may be more appropriate in certain cases.

The method for flammable gases is that required by DOT. None other was considered.

The autoignition method was also recommended by NFPA, and was evaluated in part by Affens and Carhart (1974). The method used for autoignition measurements is critical since a variability in results between methods can be great (100°C or more).

The thermal explodability method recommended (differential thermal analysis/scanning calorimetry (DTA/DSC), is a classic, simple chemistry technique yielding results that are easily interpreted. ASTM method E-474 is an apparatus calibration method that should be performed to assure the quality of the submitted DTA/DSC data. The impact explodability method for solids is that recommended by DOT and EPA (Offices of Pesticide Programs and Solid Waste). The impact explodability method for liquids was based on a critical review by DOT (1973).

(c) *Methods*—(1) *Test details.* For flash point (closed cup), ASTM D93-73, D56-75, D3278-73, D1437-64, E502-74, and CIPAC MT 12 (1970) should be

used. Cleveland or other open cup methods may be appropriate in rare situations.

For gas flammability (DOT), CFR 49, 173-300 (b) should be used.

For autoignition ASTM D2155-66 should be used. For thermal explodability (DTA/DSC), ASTM E487 should be used. For data recording E472 should be used. For calibration E537, E474 should be used.

For impact explodability, the Bureau of Explosives impact apparatus (CFR 49, 173.53 Note 4) should be used for solids. Bureau of Mines or Naval Ordnance Laboratory impact testers should be used for liquids.

(2) *Calculations and Information Which Should Be Recorded.* If the method is not one of the above, it should be described.

The flash point ±2°C should be recorded along with the atmospheric pressure.

For gas flammability, the upper and lower limits (v/v%) should be recorded.

For autoignition, the autoignition temperature (±3°C), the time lag before ignition, and the atmospheric pressure should be recorded.

For thermal explodability, a graph of ΔT(°C) or ΔE cal vs. T or t (sec. or min.) should be attached, the heating rate and calibration determination recorded, and the instrument (make and model) described.

For impact explodability, the results for the different heights and weights used should be recorded.

(d) *References.*

ASTM. 1974. *Annual ASTM Standards.* Philadelphia, PA.

Dept. of Transportation. Definition of class A explosives, CFR 49 173-53. Note 4. 1976.

A. H. Lasseigne. 1973. *Hazard Classification of Explosives for Transportation Evaluation of Test Methods,* U.S. Department of Transportation. Report # TES-20-73-2.

CIPAC Handbook. 1970. *Analysis of Technical and Formulated Pesticides.* Compiled by R. De B. Ashworth, et al. Edited by G. R. Raw. CIPAC, Ltd., Hertfordshire, England, Chapter 7, Vol. I.

National Fire Protection Association (NFPA). 1969. *Properties of Flammable Liquids and Gases.* Manual No. 325M, Boston, MA.

Proposed Resource Conservation and Recovery Act (RCRA) Hazardous Waste Criteria, Appendix B, 1978.

A-3.65 *Particle Size.* (a) *Objectives.* Particle size is quantitative measurement of the size of the crystalline or fibrous powder forms of new chemical substances. In the context of this document "particle diameter" or "particle size" refers to the effective spherical diameter of a particle (from Stokes' Law).

Particle size affects the probability of human inspiration through direct exposure, the likely point of deposition in the respiratory tract (if inhaled), and the distribution of the particle in the environment through widespread or large-volume uses (Corn, 1977).

Particles of lengths greater than 200 μm have been found in lung tissue that had been lavaged (Morgan, et al., 1978). Particles of diameter 5 μm or less are likely to be deposited in the broncheolar and alveolar regions. Therefore, particle size determinations are appropriate for compounds that are likely to form particles of 500 μm or less in length under conditions of expected use and handling.

(b) *Rationale.* The methods cited cover a wide range of sensitivities. All but one are ASTM methods. The non-ASTM method using the Sedigraph 5000 D particle size analyzer, was included because EPA has had favorable experience with it. The methods involve use of dry sieves, light microscopy, the Coulter counter, and sedimentation techniques. These methods are relatively inexpensive to perform. Electron microscopy data, of course, will be accepted. The Andreason Pipet method was considered and rejected because of disruption of the sedimentation medium caused by sampling (Irani & Callis, 1963, p. 70).

(c) *Methods*—(1) *Test details.* ASTM C690, E20-68, D1705, or D422 should be used for measuring particle size. D1366 should be used for data recording. The manufacturer's procedure for using the Sedigraph 5000 D analyzer should be used.

(2) *Calculations and Information Which Should Be Recorded.* If the method used is not one cited above, it should be described.

A graph of cumulative mass percent or percent of particles of stated size vs. log diameter (in microns) should be attached.

(d) *References.*

ASTM *An ual Book of Standards.*

Corn, Mo on. 1977. Aerosols and the Primary Air Pollutants—Nonviable Particles. *Air Pollution,* A. C. Stern (ed.) pp. 77-99.

Irani, R. R., and C. F. Callis. 1963. *Particle Size: Measurement, Interpretation and Application.* John Wiley & Sons, Inc., New York, NY.

Morgan, A., et al. 1978. Significance of fiber length in the clearance of asbestos fibers from the lung. *British J. Indust. Med.* 35: 146-153.

A-3.66 *pH Measurement.* (a) *Objectives.* Knowledge of the pH of a chemical solution is useful primarily for assessing the risk of acute hazards for health and ecological effects. Data on pH is useful for estimating eye and dermal irritation for pH values less than 3 or greater than 12. This data will also indicate possible pH changes in an aquatic system at the point of effluent discharge or in the event of a spill. Depending on the buffering capacity of the ambient waters, the effects on local aquatic life could be significant.

(b) *Rationale.* The method involves measuring the pH of chemicals in a 1% v/v concentration of chemical in water, which is estimated to be the greatest concentration in the vicinity of a large discharge. More chemicals are soluble at this concentration than at a higher concentration. For health effects assessments, the pH value so obtained can be extrapolated linearly to higher concentrations (i.e., an estimate of the pH of a 50% solution would equal pH at 1% + log 50). For compounds not soluble at 1% concentration, dioxane was chosen as a cosolvent because empirical correction factors have been determined for various concentrations of dioxane/water solutions (Irving and Mahnot, 1968). Because dioxane is a suspect human carcinogen, it should be handled in accord with "good industrial hygiene practices and procedures." One should consult D.H.E.W. (NIOSH) Pub. #77-226 *NIOSH Criteria for Recommended Standard Occupational Exposure to Dioxane.* Other organic solvent systems with pH correction factors are suitable.

Data on pH should be submitted for all solid and liquid chemicals expected to form nonneutral solutions with water or water/dioxane.

(c) *Methods*—(1) *Test details.* The pH should be measured using a 1% v/v aqueous solution of the chemical in CO_2-free distilled water. If the compound is not soluble at that concentration, a solution of purified dioxane in water may be used, with up to 80% dioxane/water (v/v). Other solvents may be used if they have an empirical correction factor for pH, such as the one for dioxane (Irving and Mahnot, 1968):

$$-\log[H^+] = B + \log U^+_H + \log f$$

where B = apparent pH, $\log U^+_H$ is an empirical correction factor at zero ionic strength, and $\log f$ is the logarithm of the mean activity coefficient of hydrochloric acid in the solvent mixture at the same temperature and ionic strength.

Glass electrode pH meters (either combination, or reference and standard pair) should be used. Standard buffer solutions at two or three pH values which bracket the expected pH of the test solution should be used for calibration. Indicator paper is not suitable.

It is not necessary to prepare fresh buffer solutions before each series of measurements, as implied by the recommended protocols: Method 144 (APHA, 1976) and ASTM E70-74. (1974).

(2) *Calculations and Information Which Should Be Recorded.* A brief description of the procedure used (if not

the same as above) should be provided, noting the pH and the temperature.

(d) *References.*

Irving, H. H., and U. S. Mahnot, 1968. pH-meter corrections for titrations in mixtures of water and dioxane. *J. Inorg. Nucl. Chem.* 30: 1215-1220.

American Public Health Association. 1974. *Standard Methods for the Examination of Water and Wastewater.* 13th ed. Washington, D.C., pp. 274-76.

A-3.67 *Chemical Incompatibility.* (a) *Objectives.* Chemical incompatibility determines the likelihood of violent reactions occurring when the new chemical is mixed or comes into contact with other substances. Thus, dangerous contact during its chemical life (manufacturing, processing, distribution, use, and disposal) can be avoided. Significant temperature increases, evolution of gases, noxious fumes, splattering, and evolution of flame are possible dangers. As used here, "significant temperature increases" is defined as an increase of 5°C or more. As with flammability/explodability properties, testing might be replaced by structure/activity analyses.

(b) *Rationale.* These tests will indicate hazardous reactions which can occur resulting from contact of the chemical with common oxidizing and reducing agents, common fire extinguishing agents, and common solvents.

(c) *Methods*—(1) *Test details.* This qualitative assessment will vary from chemical to chemical depending on physical state and intended uses. Subjective judgments by a chemist familiar with the chemical and its intended uses are needed in selecting appropriate tests. The following is only an example of an appropriate set of tests for a hypothetical chemical. All temperature increases of greater than 5°C should be noted. Tests should be conducted at temperatures expected during the normal use of the chemical. Generally, the ratio of the mass of the new chemical to the mass of the chosen reactant should be high, simulating maximum exposure situations.

(A) The chemical should be placed in contact with water for 24 hours and visual observations recorded.

(B) The chemical should be placed in contact with carbon dioxide and/or monoammonium phosphate for 24 hours, and visual observations recorded. Both of these compounds are widely used fire extinguishing agents.

(C) The chemical should be placed in contact with zinc or iron for 24 hours and observations recorded. Elemental zinc and iron are both moderately strong reducing agents. Data from corrosion tests may be suitable in lieu of this test.

(D) The chemical should be placed in contact with a moderately strong oxidizing agent, one which the chemical may contact in its intended use. The electrochemical series should be used as a guide (CRC *Handbook of Chemistry and Physics*, for example). A dilute, neutral, aqueous solution of potassium permanganate may be appropriate.

(E) If the chemical is intended for use in households, it should be placed in contact with a household organic solvent, such as kerosene, turpentine, or gasoline, for 24 hours and observation recorded.

(2) *Calculations and Information Which Should Be Recorded.* Descriptions and results of all tests should be provided.

(d) *References.*

National Fire Protection Association 1976. Extinguishing agents, *Fire Protection Handbook*, 14th ed. NFPA, Boston, MA.

A-3.68 *Vapor Phase UV Spectrum for Halocarbons.* (a) *Objectives.* In recent years a great deal of work has been done demonstrating the potential for depletion of stratospheric ozone by halogenated organic compounds (Molina and Rowland, 1974; National Academy of Sciences, 1976). This measurement may be used to make qualitative assessments of the risk posed by halogenated hydrocarbons to stratospheric ozone concentrations. Absorption cross sections can be calculated for different wavelengths based on absorbance, cell path length, and gas density of the cell. Based on this information, known structure-activity relationships, and an assumed quantum yield of 1, tropospheric half-lives can be calculated. Tropospheric half-lives of greater than several years will indicate that the chemical is likely to be transported to the stratosphere. Rowland and Molina (1975) and Chou, et al. (1978), indicate that a large fraction of chlorinated organic compounds transported to the stratosphere will release chlorine atoms either via reaction with $O(^1D)$ or via photodissociation. Therefore, any halocarbons in the stratosphere may pose a threat of reduction in ozone concentration.

This measurement is appropriate for solids, liquids, and gases that contain chlorine or bromine, and have a vapor pressure equal to or greater than 0.5 Torr at 25°C.

(b) *Rationale.* The method is a standard, simple, reproducible laboratory technique.

(c) *Methods*—(1) *Test details.* Evacuated spectral cells should be filled to predetermined gas concentrations (pressures). The spectrum should be recorded as the absorbance or molar absorptivity as a function of wavelength compared to a matched cuvette blank.

Vapor phase absorption spectra should be measured at three different pressures, between the wavelengths 275 nm and 700 nm, and at room temperature (e.g. Chou, et al., 1978; Tsubomura, et al., 1964; Gordus and Bernstein, 1954; and Lacher, et al., 1950). Calibrate the cell path length of 10 cm cells using a reference compound with an accurately known molar absorptivity and Beer's Law.

(2) *Calculations and Information Which Should Be Recorded.*

The absorbance or molar absorptivity as a function of wavelength for each concentration (gas density) should be recorded. For tabular data, wavelength intervals of two or five nanometers should be used.

The cell pathlength and the spectrophotometer model name and number, the temperature of the cell, and cell calibration data (for 10 cm cells) should be recorded.

(d) *References.*

Chou, C. C., et al. 1978. Stratospheric photodissociation of several saturated perhalochloroflurocarbon compounds in current technological use (Fluorocarbons 13, 113, 114, and 115). *J. Physical Chem.* 82: 1.

Gordus, A. A., and Bernstein, R. B. 1954. Isotope effect in continuous ultraviolet absorption spectra: Methyl bromide d, and chloroform d. *J. Chemical Phys.* 22(5): 290-295.

Lacher, J. R., et al. 1950. The near ultraviolet absorption spectra of some fluorinated derivatives of methane and ethylene. *J. American Chemical Soc.* 72: 5486-5489.

Molina, M. J., and F. S. Rowland. 1974. Stratospheric sink for chlorofluoromethanes: Chlorine atom catalysed destruction of ozone. *Nature* 249: 810-812.

National Academy of Sciences. 1976. *Halocarbons Effects on Stratospheric Ozone*, Washington, D.C.

Rowland, F. S., and M. J. Molina. 1974. Chlorofluoromethanes in the environment. *Reviews Geophysics Space Physics* 13: 1.

Tsubomura, H., et al. 1964. Vacuum ultraviolet absorption spectra of saturated organic compounds with non-bonding electrons. *Bull. Chem. Soc. Japan* 37: 417-423.

A-3.69 *Ultraviolet and Visible Absorption Spectra in Aqueous Solution.* (a) *Objectives.* The ultraviolet and visible absorption spectra of chemicals in solution are important physical properties that are characteristic of molecular structure. Since water covers most of the earth's surface, water is the obvious choice as the solvent. The ultraviolet and visible absorption spectra of chemicals in aqueous solution are also useful as an aid in carrying out and interpreting the photochemical transformation of chemicals in aqueous solution.

It may be necessary in the assessment process to obtain the ultraviolet and visible absorption spectra for chemicals that are liquids or solids at 25° C.

(b) *Rationale.* The test method outlined in Section (c) is the conventional method for determining the ultraviolet an visible absorption spectra of chemicals in aqueous solution.

(c) *Methods*—(1) *Test Details.* The following general discussion and test procedures can be used as guidelines to determine the ultraviolet and visible absorption spectra in aqueous solution.

(A) Very pure water should be used (e.g., water comparable to reagent grade water from a Millipore Milli-Q System).

(B) Spectrograde methanol or acetonitrile should be used as a cosolvent.

(C) Analytically-pure chemical should be used.

(D) If possible, the ultraviolet and visible absorption spectra of the chemical should be determined in water. However, this task is sometimes difficult, if not impossible, because the solubility of many organic chemicals in water is very low. As an alternative, mixtures of water and acetonitrile or methanol can be used as the solvent. These organic solvents have refractive indices which are very close to that of water, so the absorption spectra of nonpolar compounds will be very similar in mixtures of water and acetonitrile or methanol as in water (Zepp, 1978). The minimum amount of cosolvent required to achieve a concentration which will give a good spectrum should be used.

(E) Particular care should be used to obtain a good spectrum in the absorption "tail", since tailing absorptions may contribute significantly to the light absorption by the chemical in sunlight.

(F) When measuring the absorption spectra, a solvent vs. solvent baseline should be established, and replicates run to ensure that no instrumental or cell effects introduce artifacts into the absorption data. It is desirable to obtain molar absorption coefficients from the spectra.

(G) In some cases, cells of longer pathlength (e.g., 10 cm cells) can be used to obtain the spectrum, but must be a matched set, and must be accurately calibrated. The pathlength of these cells can be determined as follows: The Beer-Lambert equation is given by $A_\lambda = \epsilon_\lambda lC$, where A_λ is the absorbance at wavelength λ, l is the cell pathlength in cm, C is the molar concentration, and ϵ_λ is the molar absorption coefficient at wavelength λ. An analytically pure reference compound with an accurately known value of ϵ_λ should be chosen. A solution should be prepared at a molar concentration C which will give a reasonable value of A_λ in a given solvent. Before measuring A_λ, solvent vs. solvent in the two cells should be run to establish the baseline. The sample cell should be filled with the reference solution and the reference cell with the solvent. The absorbance should be measured in duplicate at the wavelength λ corresponding to the known value of ϵ_λ. The pathlength l can then be calculated using A_λ, C and ϵ. in the Beer-Lambert equation. The procedure should be repeated to calibrate the reference cell. Since the cells are a matched set, the pathlengths should be essentially the same.

(H) The ultraviolet and visible absorption spectra should be obtained at pH 5, 7 and 9 for chemicals which reversibly ionize or protonate (e.g., inorganic acids and bases, carboxylic acids, phenols, and anilines). Buffer solutions should be prepared according to the *Handbook of Chemistry and Physics* (1974-75).

(I) The molar absorption coefficient will be very useful in the interpretation of the data on photochemical transformation of chemicals in water. The molar absorption coefficient should be determined from the two absorption spectra at sufficient wavelengths to define the absorption band, including "tailing". Determining the absorption coefficient in the "tail" is difficult since it will have a low value. At wavelengths where the absorbance is indistinguishable from the baseline noise, the value of ϵ_λ should be recorded as zero.

(2) *Calculations and Information Which Should be Recorded.*

(A) The Ultraviolet and visible spectra of the chemical in aqueous solution, or in water plus methanol, or in water plus acentonitrile should be determined.

(B) The concentration of the solution and the cell pathlength.

(C) If 10 cm cells are used, submit all data on the determination of the cell pathlength.

(D) The name, structure, and purity of the reference chemical.

(E) Extinction coefficients ϵ_λ should be recorded as a function of the wavelength.

(F) The manufacturer and model number of the spectrophotometer should be recorded.

(d) *References.*

Handbook of Chemistry and Physics. 1974-1975. 55th Edition. CRC Press, Cleveland, Ohio.

Zepp, R.G. 1978. Quantum yields for reactions of pollutants in dilute aqueous solution. *Environ. Sci. and Tech.* 12:327.

A-4 Persistence Tests

A-4.1 *General.* Chemical persistence is defined here as the property of a compound to retain its physical, chemical and functional characteristics in the environments through which it is transported and distributed for a certain period of time following its environmental release. Persistence is an important aspect of a discharged chemical in the overall evaluation of the potential risk posed by the chemical to the environment.

In testing for health and ecological effects of chemicals, the range of concentrations or dosages usually employed has, as an upper limit, the minimum concentration which produces a deleterious effect. In the assessment of potential chemical risk, the allowable use of persistent chemicals must be based on an exposure safety factor derived from the dose giving a minimal effect and production data. If a chemical persists in an ecosystem and is continuously or periodically deposited there, it could accumulate to levels that exceed acceptable limits.

Chemical transformation can be considered the inverse of persistence. Transformation products can be hazardous in their own right. The fate of chemicals in the environment depends upon various chemical, physical and biological interactions. Few of these have been studied in enough detail to predict dominant pathways or rates of change in concentration. However, one intent of these Guidelines is to focus attention on persistence testing so that these deficiencies can be systematically corrected in the future.

A-4.2 *Chemical Transformation: Hydrolysis.* (a) *Objectives.* Chemicals introduced into aqueous media in the environment could undergo hydrolysis and be transformed into new chemicals with properties different from their precursors. In addition, processes other than nucleophilic displacement by water may occur (e.g., elimination or isomerization). The importance of these transformations of chemicals as dominant pathways in aqueous media can be determined quantitatively from data on hydrolysis rate constants and half-lives.

Hydrolysis data will generally be important in assessing risks from organic chemicals that have hydrolyzable functional groups (e.g., esters, amides, alkyl halides, epoxides, and phosphoric esters).

(b) *Rationale.* Hydrolysis refers to the reaction of an organic chemical (RX) with water with the resultant net exchange of the group X for the OH group from the water at the reaction center. Therefore,

$$RX + HOH \longrightarrow ROH + HX \quad . \quad (1)$$

In the environment, hydrolysis of organic chemicals occurs in dilute solution. Under these conditions, water is present in a large excess and the concentration of water is essentially constant during hydrolysis. Hence, the kinetics of hydrolysis are pseudo-first-order at a fixed pH.

Processes other than nucleophilic displacement by water can take place. For example, X can be lost from RX via an elimination reaction. These

elimination reactions exhibit kinetic behavior (i.e., pH independent or first-order acid or base dependent) similar to those reactions where OH substitution occurs. Elimination reactions are also covered in this hydrolysis protocol.

The hydrolysis reaction can be catalyzed by acidic or basic species, including OH^- and H_3O^+ (H^+). The promotion of hydrolysis by H^+ or OH^- is known as specific acid or specific base catalysis, as contrasted to general acid or base catalysis encountered with other cationic or anionic species. So far, the published laboratory data (e.g., Smith, et al., 1977, 1978; Wolfe, et al., 1977; Zepp, et al., 1975) indicate that hydrolysis rates are the same in sterile natural freshwaters and in buffered distilled water at the same temperature and pH. Thus, only specific acid or base catalysis together with neutral water reaction need be considered. Although other chemical species may catalyze hydrolysis reactions, the available concentrations of these species in the environment are usually too low to have an effect and are not expected to contribute significantly to the rate of hydrolysis (Mabey and Mill, 1978).

An extensive amount of information has been published on the hydrolysis of a wide variety of organic chemicals. However, most of the literature relating to environmental hydrolysis of chemicals pertains to pesticides. Much of this data is incomplete for the range of pH and temperature of environmental concern. Effects of buffer salts are often unrecognized. At present, no published screening test exists for determining the hydrolysis rate constant at relevant environmental pHs and temperatures. Such a test is being developed. In the interim, the general discussion and test procedures described herein can be used as guidelines. These procedures take into account (1) the fundamentals of the kinetics of hydrolysis; (2) the need for quantitative data in the pH and temperature ranges of environmental concern; (3) the effects of buffer salts on the rates of hydrolysis; and (4) the need to assure that only hydrolysis takes place. For example, the experiments should be designed to prevent biodegradation, loss by volatilization, or photolysis (for chemicals that absorb in the visible region).

(c) *Methods*—(1) *Test Details.* The following general discussion and test procedures can be used as guidelines for determining the hydrolysis of organic chemicals.

(A) Hydrolysis experiments should be conducted in a thermostatic bath with good temperature control (±1°C) and with accurate temperature measurement.

(B) Hydrolysis experiments should be performed using analytically-pure material (greater than 99% by weight pure), if available. However, if the chemical contains some impurities, note the nature and amount (%) of each impurity.

(C) Very pure, sterile water should be used (e.g., water comparable to reagent grade water from a Millipore Milli-Q-System).

(D) All glassware should be sterilized in an autoclave or by some other suitable method.

(E) Buffer solutions should be prepared using reagent grade chemicals and very pure water, as follows:

pH 5.0: 0.01M sodium acetate adjusted to pH 5.0 with 0.1M acetic acid.
pH 7.0: 30 ml of 0.067M NaH_2PO_4 mixed with 61 ml of 0.067M K_2HPO_4 and diluted tenfold.
pH 9.0: 0.025M $Na_2B_4O_7$ adjusted to pH 9.0 with 0.1M acetic acid.

The pH of each buffer solution should be checked with a pH meter at 25°C and adjusted to pH 5.0, 7.0, or 9.0, if necessary. These solutions should be used to prepare reaction solutions of the chemical.

(F) Test solutions should be prepared as follows:

(i) If the chemical is readily soluble in water, an aqueous solution of chemical in very pure water should be prepared, with buffer solution added to an aliquot of this aqueous solution. The chemical concentration in the final solution should be less than one-half its solubility in water and less than 10^{-2}M.

(ii) For chemicals which are too insoluble in pure water to permit reasonable handling and analytical procedures, pure chemical should be dissolved in reagent grade acetonitrile and buffer solution should be added to an aliquot of the acetonitrile solution. The chemical concentration in the final solution should be less than one-half its solubility in water and less than 10^{-2}M. The concentration of acetonitrile should be one volume-percent or less, in the final solution.

(G) The solution absorption spectrum, as measured by the method described in Section A-3.69, can be used to determine whether the hydrolysis experiments should be carried out in the dark to eliminate photolytic transformations. For chemicals that absorb in the visible region, the experiments should be conducted using amber or red safelights, amber or red glassware, or some other suitable method.

(H) Stoppered volumetric flasks (no grease) should be used for carrying out the hydrolysis reaction. If the chemical is volatile, the tubes should be sealed. Tubes should be filled almost completely, sealed, and submerged in the constant-temperature bath.

(I) Any suitable analytical procedure can be used for the determination of the concentration of chemical at various times during the course of the hydrolysis reaction. Whenever possible, an analytical procedure that provides a precision within ± 5% should be used.

(J) Complications can be encountered when measuring the rate of hydrolysis of compounds that reversibly ionize or protonate in the pH range 5 to 9. Rates of hydrolysis of these compounds often show unusual pH-rate profiles, often with a maximum or minimum, owing to competition between charged and uncharged forms. Mabey and Mill (1978) depict typical log k_h vs pH plots for chemicals which undergo acid, water, and base promoted hydrolysis (Figure 1). Should there be any question about the importance of this effect on the hydrolysis of compounds that reversibly ionize or protonate in the pH range 5–9, additional measurements should be made to define the pH-rate profile.

(K) In general, with low concentrations of chemicals, buffers may be used at 0.01M concentrations to hold pH constant without introducing buffer effects (Mabey and Mill, 1978). The buffers outlined in (E) should be adequate. However, for some chemicals, it is possible that buffer catalysis could affect the rate of hydrolysis. If there is any reason to suspect buffer catalysis, hydrolysis experiments can be carried out at two buffer concentrations using the buffers in (E) and the same buffers at a lower concentration. If the rates of hydrolysis are the same, then buffer catalysis has been eliminated. A good discussion of general acid and base catalysis is given by Jencks (1969).

(L) As a general guideline to carrying out hydrolysis experiments, the methods of Wolfe et al. (1976); Wolfe et al. (1977); Smith et al. (1977) (1978); Barrow (1966); and Frost and Pearson (1961) should be followed. All experiments should be conducted in duplicate by one of the following procedures.

Reaction solutions of the chemical should be prepared as described above at 25°C at pH 5.0, 7.0, and 9.0. The initial concentration (C_o) should be determined at t=0.

Procedure 1: Each reaction mixture should be analyzed at regular time intervals to provide a minimum of six time points (in hours) with hydrolysis between 20–70%. Rates should be fast enough so that 60–70% of the chemical is hydrolyzed in four weeks (672 hours).

Procedure 2: If hydrolysis is too slow at 25°C to conveniently follow the reaction to high conversion in four weeks but still rapid enough to measure 20–30% conversion, the accuracy

of the data will be impoved if more data points are taken between 10 and 30% conversion. Fifteen to twenty time points are preferable.

Procedure 3: If the chemical hydrolyzes less than 20% in four weeks, the concentration (C) should be determined after four weeks (t=672 hours).

(2) *Calculations and Information Which Should Be Recorded.*

(A) A description of the analytical procedure and data on calibration should be recorded.

(B) A description of the method of extraction and data on recovery should be recorded if an extraction method is used to separate the chemical from the aqueous phase.

(C) The initial concentration of the chemical should be recorded for each set of experiments.

(D) The pH for each set of experiments should be recorded.

(E) Hydrolysis calculations should be carried out in the following manner. Since the kinetics of hydrolysis is pseudo-first-order, the integrated equation can be put in the form

$$\log_{10}C = -\left[\frac{k_h}{2.30}\right] t + \log_{10}C_o \quad , \quad (2)$$

C_o is the initial concentration of the chemical, C is the concentration of chemical at t (in hours) and k_h is the hydrolysis rate constant (in hours $^{-1}$) at a fixed temperature and pH. Using the experimental data from Procedures 1 or 2 and linear regression analysis, k_h can be accurately determined at 25° C. k_h and the correlation coefficient (R^2) for each set of experiments should be calcluated and recorded. The mean value of k_h and the standard deviation should be calculated and recorded. From the mean value of k_h at pH 5.0, 7.0, and 9.0 at 25° C, the half-life $t_{1/2}$ can be calculated using the following equation:

$$t_{1/2} = \frac{0.693}{k_h} \quad (3)$$

The values of $t_{1/2}$ at pH 5, 7, and 9 should be calculated and recorded. For Procedure 3, the initial concentration C_o and the concentration of chemical C remaining at t=672 hours should be used in equation 2 and k_h calculated at 25° C for each set of experiments. The mean value of k_h and the standard deviation at pH 5, 7, and 9 should be calculated and recorded. From the mean value of k_h at pH 5.0, 7.0, and 9.0 at 25° C, the half-life, $t_{1/2}$ should be calculated and recorded. If after 672 hours, C is essentially equal to C_o, then k_h cannot be calculated and the chemical would then be considered persistent.

(d) *References.*

Barrow, G. M. 1966. *Physical Chemistry,* McGraw-Hill, New York, N.Y.

Frost, A. A. and R. G. Pearson. 1961. *Kinetics and Mechanism.* Second Edition. John Wiley & Sons. New York, N.Y.

Jencks, W. T. 1969. *Catalysis in Chemistry and Enzymology.* Chapter 3. McGraw-Hill. New York, N.Y.

Mabey, W. and T. Mill. 1978. Critical review of hydrolysis of organic compounds in water under environmental conditions. *J. Phys. and Chem. Ref. Data* 7:383.

Smith, J. H. et al. 1977. *Environmental Pathways of Selected Chemicals in Freshwater Systems. Part I. Background and Experimental Procedures.* EPA-600/7-77-113.

Smith, J. H. et al. 1978. *Environmental Pathways of Selected Chemicals in Freshwater Systems. Part II. Laboratory Studies.* EPA-600/7-78-074.

Wolfe, N. L. et al. 1976. *Chemical and Photochemical transformation of Selected Pesticides in Aquatic Systems.* EPA-600/3-76-067.

Wolfe, N. L. et al. 1977. Kinetics of chemical degradation of malathion in water. *Environ. Sci. Technol.* 9:88.

Zepp, R. G. et al. 1975. Dynamics of 2,4-D esters in surface waters: Hydrolysis, photolysis, and vaporization. *Environ. Sci. Technol.* 9:1144.

A-4.3 *Chemical Degradation: Oxidation.* (a) *Objectives.* For many organic chemicals which are gases at ambient temperatures or which have appreciable volatility, atmospheric oxidation undoubtedly is a major degradative pathway. A number of studies of these reactions, principally in air pollution research, make predictions possible on the fate of a variety of organic compounds in the atmosphere. Oxidants produced *in situ* in the air through photochemical oxidation include ozone, hydroxy radicals, peroxides, hydroperoxides and peroxy nitrates. The most important oxidants of organic vapors are the hydroxy radicals and ozone.

Chemical oxidation occurs in aquatic environments as a consequence of photochemical processes that generate free radicals. The concentrations and reactivities of oxy free radicals are such that only alkyl-peroxy radicals and singlet molecular oxygen are likely to act as important oxidants in natural waters (T. Mill, 1978).

Data from free radical oxidation studies will permit the calculation of estimated half-lives and degradation rates and the assessment of oxidation as a factor in the environmental persistence of the test chemical.

Chemicals subject to rapid atmospheric oxidation are less likely to persist and be transported from the point of release. On the other hand, a chemical not likely to be rapidly oxidized or degraded by other mechanisms is more likely to have an effect on various life forms.

There are some indications that free radical oxidations may be important only for certain types of organic chemicals, but this needs more study. Until applicability can be limited on the basis of structure-activity relationships, the free radical oxidation methods apply as follows:

(1) For atmospheric oxidations, testing with hydroxy radical and with ozone for all organic chemicals except those with vapor pressures of less than 10 torr at 30°C.

(2) Oxidation testing in water for all organic chemicals except those with a water solubility of less than 0.1 mg/l at 20°C.

(b) *Rationale.* Free radical oxidations are important degradative mechanisms for many organic compounds in the atmosphere and such reactions may be important for some organic chemicals in water and soil. However, there are at present no simple procedures for evaluating the potential for free radical oxidations in the environment. Thus, selected references will be cited that describe typical methods currently used to measure the oxidation of organic compounds at environmentally realistic concentrations. Development and validation of procedures acceptable to EPA will be based on these methods.

Appropriate studies of free radical oxidation in air include enclosure of the reactants—an oxidant species and the organic compound—in a container at appropriate concentrations. Analytical procedures are used to follow the change in concentrations of one or both of the reactants.

Some technology is being developed for determining the free-radical oxidation potential in water (Smith, et al., 1977). In this method alkylperoxy radicals are generated at a known concentration in water and the disappearance of an organic test compound is followed. The procedures need further development and refinement, and methods need to be developed for testing for oxidation by singlet oxygen. It also appears that only selected classes of chemicals will be sufficiently reactive with free-radical oxidants to justify testing. Those classes need clearer definition.

There is no technology for the study of free-radical oxidations in soil.

The referenced procedures are the best state-of-the-art methods for determining free-radical oxidation under environmental conditions.

(c) *Methods.* The cited procedures measure the potential for a chemical to undergo free-radical oxidation in airborne or aqueous environments, but they require modifications. Until test details are developed and validated, the following references should be used as guidelines.

(1) *Atmospheric Studies.* The important oxidant species in the air appear to be ozone and hydroxy radical. Tests with ozone may be performed as described by Doyle et al. (1975), Japer et

al. (1974) or Walter et al. (1977). An example of good studies of hydroxy radical reactions is described by Niki et al. (1978).

(2) *Oxidation in Water.* Methodology currently under development is described by Smith et al. (1977).

(3) *Calculations and Information Which Should be Recorded.* (A) A written description of the method used (or equivalent literature reference) should be prepared, including details on equipment, analytical techniques, reactant species and concentrations, and reaction conditions (temperature, light, etc.).

(B) The half-life calculations and results for the organic test compound should be recorded.

(C) A written discussion of any difficulties experienced should be included along with any other pertinent information.

(d) *References.*

Doyle, G.J. et al., 1975. Gas phase kinetic study of relative rates of reaction of selected aromatic compounds with hydroxyl radicals in an environmental chamber. *Env. Sci. Tech.* 9:237-241.

Hill, James IV, et al., 1976. Dynamic behavior of vinyl chloride in aquatic ecosystems. A report for the Office of Research and Development, U.S. EPA, Athens, GA. EPA-600/3-76-001.

Japer, S.M., et al., 1974. Rate constants for the reaction of ozone with olefins in the gas phase. *J. Phys. Chem.* 78:2318-2320.

Mill, R. Unpublished.

Niki, H. et al., 1978. Relative rate constants for the reaction of hydroxyl radical with aldehydes. *J. Phys. Chem.* 82:132-134.

Smith, J.H., et al., 1977. Environmental pathways of selected chemical in freshwater systems. A report for EPA, Office of Research and Development, Athens, GA. EPA Report No. 600/7-77-113.

Walter, T.A., et al., 1977. Mechanism for olefin-ozone reactions. *Env. Sci. Tech.* 11:382-386.

A-4.4 *Photochemical Transformation in Water.* (a) *Objectives.* Chemicals introduced into aqueous media in the environment can undergo transformation by direct photolysis in sunlight into new chemicals with different properties than their precursors. Data on direct photolysis rate constants and half-lives establish the importance of direct photolysis in sunlight as a dominant transformation of chemical in aqueous media. Therefore, risk assessment may demand photolysis data on chemicals that are liquids or solids at 25°C. except aliphatic hydrocarbons including alkanes, alkenes, and alkynes.

(b) *Rationale.* Although numerous papers have been published on the photolysis of chemicals in solution, rate constants for direct photolysis of chemicals in water under environmental conditions (i.e., in sunlight) have emerged only in the last three years. Zepp and Cline (1977) published a paper on photolysis in aquatic environments with equations for direct photolysis rates in sunlight. These equations translate readily obtained laboratory data into rate constants and half-lives for photolysis in sunlight. Rate constants and half-lives can be calculated as a function of season, latitude, time-of-day, depth in water bodies, and the ozone layer. Several published papers concerning the photolysis of chemicals in the presence of sunlight verify this method (Zepp, et al., 1977; Wolfe, et al., 1976; Zepp, et al., 1976; Zepp, et al., 1975; Smith, et al. 1977, 1978).

This preliminary test is based on the following criteria: (1) the basic fundamentals of photochemistry; (2) the need for quantitative data on direct photolysis rates of organic chemicals in aqueous media; (3) the use of sunlight as the irradiation source because of its relevance and low cost in comparison to artificial light sources; and (4) the experimental design so that only photolysis occurs. For example, the experiments should be designed to eliminate biodegradation and volatilization. However, the test method fails to measure sunlight intensities on the sample during photolysis. Reference compounds (i.e., sunlight actinometers) will be developed through EPA contract to evaluate sunlight intensities. The test will then be modified to use simultaneous photolysis of a chemical and actinometer to evaluate sunlight intensities on the sample. The mofidied procedure will quantify sunlight photolysis of a chemical at a specific time of year and latitude and gives a less accurate but still useful measure of seasonal variation of photolysis.

Substances present in natural fresh waters may accelerate or retard the photolysis of chemicals in sunlight. How these substances affect photolysis is not completely understood. Research is needed to determine what effects substances present in natural waters may have on photolysis rates, with emphasis on the role of various sensitized and photo-initiated free radical processes in accelerating photolysis. Research is also needed on the effect natural substances may have on quenching or retarding photochemical processes. Therefore, only very pure air-saturated water is recommended for use in the screening tests.

(c) *Methods*—(1) *Test details.* Use the following discussion and test procedures as guidelines for determining the photolysis of chemicals in aqueous solution.

(A) conduct photolysis experiments on an analytically pure chemical (greater than 99% by weight pure), if available. If the chemical contains some impurities, specify the nature and amount (%) of each impurity.

(B) Use very pure, sterile, air-saturated water (e.g., water comparable to reagent grade water from a Millipore Milli-Q-System).

(C) Sterilize glassware in an autoclave or by another suitable method.

(D) Prepare solutions with the chemical (1) at less than one-half its solubility in water, (2) at less than 10^{-5} M, and (3) at a concentration such that the absorbance is less than 0.02 in the reaction cell at wavelengths greater than 290 nm. Since the reaction cell is approximately 1 cm, the absorbance of the solution should be less than 0.02 as measured spectrophotometrically in a 1 cm cell. For chemicals which are too insoluble in pure water to permit reasonable handling and analytical procedures, prepare reaction solutions from pure chemical dissolved in reagent grade acetonitrile, an aliquot of which is added to very pure water. Do not exceed one volume-percent of acetonitrile in the final solution.

(E) Carry out photochemical studies at pH 5, 7, and 9 with chemicals which reversibly ionize or protonate (e.g., inorganic acids and bases, carboxylic acids, phenols, and anilines). Prepare buffer solutions by procedures given in the hydrolysis protocol.

(F) The absorption spectrum, as measured by the method described in Section A-3.69, will determine what type of reaction cell can be used in the photolysis experiments. For chemicals that absorb below 340 nm, use approximatly 11 mm i.d. quartz ground-glass stoppered tubes (no-grease). Chemicals that absorb at wavelengths greater than 340 nm can be tested in borosilicate tubes. Cylindrical borosilicate reaction tubes with an i.d. of 11 mm are acceptable. Tubes comparable to Corning Glass, Inc., disposable culture tubes (13 X 100mm) are convenient because they have screw caps for easy sealing. Fill almost completly either tube type with the reaction solution.

(G) Use any suitable analytical method for the determination of the concentration of chemical at various times during the course of the photolysis. Whenever possible, use an analytical procedure that provides a precision within ±5%.

(H) Zepp and Cline (1977) published a paper on determining the rates of direct photolysis in aquatic environments, where the rate of decrease of the concentration of the chemical (C) with time was given by equation 1:

$$-\frac{dC}{dt} = \phi k_a C = k_p C \qquad (1)$$

$$k_p = k_a \phi \quad , \qquad (2)$$

where ϕ is the reaction quantum yield of the chemical in dilute aqueous solution, and $k_a = \sum k_{a\lambda}$, the sum of $k_{a\lambda}$ values of all wavelengths of sunlight that are absorbed by the chemical. The term k_p represents the photolysis rate constant in sunlight. Integrating equation 1 yields:

$$\log_{10} \frac{C_o}{C} = \frac{k_p t}{2.30} \quad , \quad (3)$$

where C is the molar concentration of chemical at time t during photolysis and C_o is the initial molar concentration.

By measuring the concentration of chemical C as a function of t during photolysis in sunlight, k_p can be determined using equation 3. Because equation 1 is a first-order rate equation, the half-life for direct photolysis in sunlight is given by equation 4:

$$t_{1/2} = \frac{0.693}{k_p} \quad (4)$$

Zepp and Cline (1977) compute (by computer) direct photolysis rate constants in sunlight (k_p) using a series of equations and laboratory data. Zepp (1978) also determined the quantum yield ϕ by laboratory methods.

(I) For details on conducting photolysis experiments in water, consult the reports by Smith, et al. (1977, 1978) and Wolfe, et al. (1976). The following procedure is a general guideline for determining k_p in sunlight, as an average value for a 24-hour day (expressed in units of day $^{-1}$). For all experiments, prepare an aqueous solution of the chemical and a series of samples in quartz tubes or in sealed 11 mm i.d. borosilicate tubes, as described above in duplicate. Prepare two control samples by wrapping the tubes with aluminum foil. These are analyzed for chemical immediately after completion of the experiment to make sure no decomposition occurred. Place the samples, including the controls, outdoors in an area free of shade and reflections of sunlight from windows and buildings on a black background and inclined at approximately 30° from the vertical. Conduct the photolysis experiments during a warm time of year (i.e., June, July, August) and begin before sunrise. Record the date begun and completed and the latitude of the site. Use one of the following four procedures, depending on how fast the chemical photolyzes:

(i) *Procedure 1:* If the chemical photolyzes 60–80% in 28 days, measure the concentration of chemical at time t = 0 and periodically at 12 o'clock noon.

(ii) *Procedure 2:* If the chemical photolyzes in the range 20–50% in 28 days, determine the concentration after 28 days of photolysis. If less than 20% of the chemical photolyzes after 28 days of exposure, the chemical has a half-life in water greater than 3 months.

(iii) *Procedure 3:* For chemicals that photolyze 60–80% in sunlight within 7 days, determine the concentration at time t=0 and at 12 o'clock noon each day.

(iv) *Procedure 4:* For chemicals that photolyze in sunlight 60–80% within one or two days, place the samples outside before sunrise and measure the concentration of chemical at t=0 and after sunset the first day and after sunset the second day. The above experiment will be carried out on cloudless days a total of three times.

(2) *Calculations and Information Which Should be Reported.* In describing the analytical method used, give the detailed procedure and all the calibration data. In addition, if an extraction method is used to separate the chemical from the aqueous phase, report the detailed method of extraction and recovery data.

For chemicals that photolyze 60–80% in 28 days, use one concentration C which corresponds to less than 40% remaining and the corresponding time in days along with the initial concentration C_o in equation 3 and calculate k_p in days^{-1}. From the analysis of duplicate samples, calculate two values of k_p. Report the mean value of k_p and the standard deviation. Calculate the half-life $t_{1/2}$ using the mean value of k_p in equation 4.

For chemicals that photolyze, 20–50% in 28 days, use the concentration C remaining at t=28 days long with C_o to calculate k_p. Use the same procedure as described above to calculate and report a mean value of k_p, the standard deviation, and $t_{1/2}$.

For chemicals that photolyze in the time period described in Procedure 3, use one concentration C which corresponds to less than 40% of chemical remaining and the corresponding time in days in equation 3 along with C_o to calculate k_p in days $^{-1}$. Calculate two values of k_p from the analysis of duplicate sample and report the mean value and standard deviation of k_p. Use the mean value of k_p in equation 4 to calculate $t_{1/2}$.

For Procedures 1, 2, and 3, if exposure has been intermittent, submit the dates and times of actual exposure. Also report latitude, site of photolysis, and unusual weather conditions.

For chemicals that photolyze rapidly as described in Procedure 4, calculate a full-day k_p value, using the concentration of chemical remaining after sunset along with C_o in equation 3. Calulate two values of k_p from the analysis of duplicate samples and report the mean and standard deviation of k_p. Repeat these calculations for the three separate full-day photolysis experiments. Calculate, and report, the half-life $t_{1/2}$ using the mean value of k_p in equation 4. Also report the dates of photolysis, the latitude, and the site.

(d) *References.*

Smith, J.H., et al. 1977. *Environmental Pathways of Selected Chemicals in Freshwater Systems. Part. I Background and Experimental Procedures.* EPA-600/7-77-113.

Smith, J.H., et al. 1978. *Environmental Pathways of Selected Chemicals in Freshwater Systems. Part II. Laboratory Studies.* EPA-600/7-78-074.

Wolfe, N. L., et al. 1976. *Chemical and Photochemical Transformations of Selected Pesticides in Aquatic Systems.* EPA-600/3-76-067.

Zepp, R. G. and D. M. Cline. 1977. Rates of direct photolysis in aquatic environment. *Environ. Sci. and Tech.* 1:359.

Zepp, R. G. 1978. Quantum yields for reactions of pollutants in dilute aqueous solution. *Environ. Sci. and Tech. 12:327.*

Zepp, R. G., et al. 1977. Photochemical transformation of the DDT and methoxychlor degradation products, DDE and DMDE, by sunlight. *Arch. Environ. Contam. Toxicol.*, 6:305.

Zepp, R. G., et al. 1976. Light-induced transformations of methoxychlor in aquatic systems. *J. Agri. Food Chem.*, 24:727.

Zepp, R. G., et al. 1975. Dynamics of 2,4-D esters in surface waters. Hydrolysis, photolysis, and vaporization. *Environmental Science and Technology*, 9:1145.

A-4.5 *Biodegradation.* (a) *Objectives.* The major use of data on biodegradation is for assessing the persistence of a chemical substance in a natural environment. The natural environment, for the purpose of such tests, is natural waters and various soils (including hydrosoils). If the compound does not persist, information is needed on whether the compound degrades to innocuous molecules or to relatively persistent and toxic intermediates. Secondary concerns for environmental persistence are the possibilities that (1) toxic substances may interfere with the normal operation of biological waste treatment units or (2) toxic substances not substantially degraded within a treatment plant may be released to the natural environment.

The test procedures for biodegradation give an estimate of the relative importance of biodegradability as a persistence factor. The data will be used to evaluate biodegradation rates in comparison with standard reference compounds.

The reference tests described below provide opportunities for biodegradation with relatively dense microbial populations which have been allowed to adapt to the test compound. Those compounds which degrade rapidly (in comparison with reference compounds) and extensively (as judged by such evidence as CO_2 evolution and loss of dissolved organic carbon) are

likely to biodegrade rapidly in a variety of environmental situations.

Such compounds may persist in specialized environments and under circumstances which are poorly represented by these preliminary tests. Further testing may be necessary which focuses on those circumstances. Compounds which produce little indication of biodegradation in these tests may be relatively persistent in a wide variety of environments. In such cases, assessment should focus on other degradation routes, transport and effects.

Reliable conclusions about biodegradation are not generally possible on the basis of structure alone. Biodegradation is the most important degradative mechanism for organic compounds in nature, in terms of mass of material transformed and extent of degradation. Therefore, information on biodegradability is very important in any evaluation of persistence and is generally needed on organic compounds that can be solubilized or dispersed in or on water. Highly insoluble compounds are not testable at present without the use of radioisotopes or complex analytical measurements, nor are there methods to study biodegradation with very low substrate concentrations. Research is needed in these areas to assure more comprehensive and representative assessment.

These types of tests do not differentiate all chemical compounds as relatively nonbiodegradable or rapidly and extensively biodegradable. Results for many materials lead to intermediate conclusions. A more complete understanding of the biodegradability of such compounds would result from advanced tests, such as those employing radiolabelled substrates. Any decision to proceed with such advanced testing would depend upon other factors, such as other transformations and adverse effects of the compound and its degradation products.

(b) *Rationale.* Relatively expensive procedures, such as the use of radiolabelled compounds or instrumental analysis for parent compound and degradation products, are avoided by using methods which follow degradation by indirect methods. These methods include measuring the production of CO_2 and/or methane, the uptake of oxygen and the disappearance of dissolved organic carbon (DOC). Among the methods are a shake flask procedure and CO_2 and BOD measurement. The procedure described by Gledhill (1975) may be substituted for these tests. Other tests are an activated sludge test and an anaerobic test. The proposed tests are based on widely used methods and are being considered as models for standard procedures. They represent the best current state-of-the-art, although each has problems which research might resolve.

The shake flask method is conducted in a mineral salts basal medium with a weak inoculum and relatively low test substrate concentration. This serves as a simple model of surface water. Significant loss of DOC during the test indicates degradation of parent compound but does not prove complete conversion of the organic molecule to CO_2, inorganic salts, and compounds involved in the normal metabolic processes of microorganisms. Loss of DOC could result from cellular uptake, sorption or loss by evaporation.

The CO_2 evolution and standard BOD tests also simulate surface water and provide an indirect measure of biodegradation. When an organic compound is degraded by microorganisms in the presence of molecular oxygen, it may be converted completely to CO_2 and inorganic salts. The theoretical maximum utilization of O_2 and evolution of CO_2 can be calculated if the carbon atoms of an organic compound are completely oxidized to CO_2. In practice, the complete mineralization of the organic substrate is seldom observed because microorganisms generally assimilate from 5 to 40% of the substrate carbon and only 60 to 95% is converted to CO_2. Thus, CO_2 evolution values (or O_2 uptakes) which exceed 60% of the theoretical maximum may indicate substantial degradation of the parent compound.

The activated sludge method simulates conditions in an activated sludge waste treatment plant with moderate to heavy concentration of activated sludge organisms and test compound. The compound to be studied is used at concentrations of 50 to 100 mg/l as DOC. These concentrations allow for analytical reliablity (more easily measured) and for blank organic carbon values which make up only a small part of the total analytical value.

Anaerobic microbial degradation of organic compounds is an important mechanism for degrading waste materials in both the natural environment and in waste treatment plants. However, there are few relatively simple state-of-the-art methods at present for evaluating the potential for anaerobic biodegradations. The types of methods most frequently cited employ microcosms such as flooded soils in flasks and require the use of radiolabelled substrate. Methane from fermentation of organic substrates is the end of a food chain process involving a wide variety or anaerobic bacteria (Mink and Dugan, 1977). The anaerobic digestion test compares the production of methane and CO_2 by anaerobic bacteria in sludge samples with and without added test material.

A desirable goal of degradation testing is to obtain some estimate of the rate at which a compound will degrade in the environment. It is relatively easy to estimate reaction rates for such degradation processes as hydrolysis, photolysis and free-radical oxidations. Environmentally realistic reaction rates for biodegradation are much more difficult to obtain. Among the more important environmental variables which can effect the rate and the extent of biodegradation are (1) temperature, (2) pH, (3) salinity, (4) dissolved oxygen, (5) concentration of test substance, (6) concentration of viable microorganisms, (7) quantity and quality of nutrients (other than test substances), trace metals, and vitamins, (8) time and (9) microbial species. The screening tests proposed in these guidelines do not attempt to deal with all of these variables and the data will not yield quantifiable information on reaction rates. The methods cited will provide indirect evidence for biodegradation, as opposed to the direct analysis for the parent compound and any intermediates that might be formed. The methods cited will provide a means to identify those compounds which are likely to biodegrade rapidly and extensively under conditions that are generally optimal for biodegradation and, also, those compounds which do not biodegrade significantly under those conditions. The methods cited will provide some means for estimating the environmental biodegradability of the test compound by including, in each test, a well-characterized reference compound. If suitable analytical methods are available for the analysis of the parent compound, those methods also may be used to follow the persistence or disappearance of that compound. The rate of disappearance of the parent compound and/or the rate of production of microbial biomass may be followed, in the shake flask test or the CO_2-evolution test, to provide some quantification of the biodegradation process. However, it is believed that such quantification is best left for subsequent tests employing radiolabelled compounds or other suitable analytical techniques which allow the use of substrate concentrations, microbial densities and the like which are more realistic representations of the environment than are the screening tests. The need for subsequent testing should be based on other factors besides biodegradability, such as other degradation/transformation possibilities and indications of adverse effects of the compound and its degradation products.

The investigator may choose to substitute more complex procedures, or procedures using appropriately radiolabelled compound (for example, the

procedures cited in the EPA Pesticides Guidelines) for the procedures described here. Such substitution is acceptable if the methods employed are commensurate with the intended conditions of chemical use and release to the environment and if the same broad environmental circumstances (aerobic aquatic, anaerobic, and activated sludge) are investigated.

There are dozens of alternative methods which could have been specified, many described in a review by Howard, et al. (1975). The proposed methods were preferred over the alternatives because of wider acceptability in the scientific community, simplicity and low cost. These methods represent the best state-of-the-art methods for biodegradability and are being considered as models for standard procedures. However, none is completely acceptable and each should use standard reference compounds concurrently with the test compound. Any problems which appear to be associated with toxicity to the microbial populations, volatility of the test compound, or nonbiological transformations should be recorded.

Sparingly soluble and insoluble materials present special problems in biodegradability tests. Solids should be pulverized or ground to powder or fine particles, if possible, and dispersed into the systems using a minimal volume of organic solvent, if absolutely necessary. When a solvent must be used, a solvent blank (a duplicate setup with everything except the test compound) should be included in the test.

(c) *Test Description.* Descriptions of tests are given at the beginning of each section on specific methods.

A-4.51 *Shake Flask Method.* (a) *Test Description.* The shake flask procedure for biodegradability is based on the Presumptive Test of the Soap and Detergent Association (1966) and the Modified OECD Screening Test (1971).

Microorganisms are inoculated into flasks that contain a defined microbial growth medium (basal medium) and the test compound. Aeration is accomplished by continous shaking of the flask. Following four adaptive transfers, biodegradation is determined by measuring the reduction (if any) in dissolved organic carbon (DOC) during the test period.

The investigator may choose to combine the shake flask method (method A-4.51) with the CO_2 evolution test (method A-4.54) using the apparatus and procedures described by Gledhill (1975). If this option is chosen, the same information should be reported with respect to DOC loss and CO_2 evolution as wouuld be the case of those measurements were made using separate procedures. The microbial inoculum source and acclimation period should be the same as for the shake flask method.

(b) *Basal Medium.* The following basal medium should be used:

(1) Water: high quality water (ASTM Type II or better, ASTM, 1974) containing less than 1 mg/l total organic carbon (TOC), from a block tin or all glass still.

(2) Phospate Buffer Solution: Dissolve 8.5 g KH_2PO_4; 21.75 g K_2HPO_4; 33.4 g $Na_2HPO_4 \cdot 7H_2$; and 15.0 g NH_4Cl, in about 500 ml of distilled water and dilute to 1 liter.

(3) Magnesium sulfate Solution: Dissolve 22.5 g $MgSO_4 \cdot 7H_2O$ in distilled water and dilute to 1 liter.

(4) Calcium Chloride Solution: Dissolve 27.5 g anhydrous $CaCl_2$ in distilled water and dilute to 1 liter.

(5) Ferric Chloride Solution: Dissolve 0.25 g $FeCl_3 \cdot 6H_2O$ in distilled water and dilute to 1 liter.

(6) Trace Element solution: Dissolve 39.9 mg $MnSO_4 \cdot 4H_2O$, 57.2 mg H_3BO_3, 42.8 mg $ZnSO_4 \cdot 7H_2O$ and 34.7 mg $(NH_4)_6Mo_7O_{24}$ in distilled water and dilute to 1 liter.

(7) yeast Extract Solution: Dissolve 15 mg of Difco yeast extract in 100 ml distilled water. Prepare immediately before use.

(8) To each liter of water, 1 ml of each solution above should be added except the yeast extract solution. The solution should be dispensed in 500 ml or 1000 ml portions into 1-liter or 2-liter narrow mouth Erlenmeyer flasks. The flasks should be stoppered with cotton plugs or the equivalent to reduce evaporation and contamination. The flasks should be sterilized if not used on the day of preparation, by autoclaving at 120°C for 20 minutes. Immediately before use, 1 ml of yeast extract per liter of solution should be added to each flask.

(c) *Microbial Culture.* The microbial culture used as the initial inoculum should be prepared as follows:

(1) Secondary Effluent Culture: A sample of secondary effluent of good quality should be obtained from a sewage treatment plant dealing with a predominantly domestic sewage, and filtered through a glass wool pad. The filtrate should be retained.

(2) Soil Culture: 100 g of garden soil (not sterile) should be obtained and suspended in 1 liter of chlorine-free tap water. Soils that are largely clay, sand, or humus should not be used. The suspension should be stirred to thoroughly mix the contents and to break up any clumps. The solids should be allowed to settle for 30 minutes. The supernatant should be filtered through a glass wool pad, and the filtrate retained.

(3) Mixed Culture Inoculum: 100 ml of secondary effluent filtrate should be mixed with 50 ml of soil suspension filtrate and used to inoculate the shake flasks within 24 hours of the time of collection of the secondary effluent and soil. Up to 25 ml of any other mixed culture suspension may be added to the 150 ml of sewage and soil organism suspension. If this is done, information on source of organisms, handling prior to use, and rationale for addition should be recorded.

(d) *Linear Alkylate Sulfonate (LAS).* A sample of LAS (Standard LAS may be obtained from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.) should be obtained. The quantity containing 10 mg of organic carbon should be calculated based on the percent of active LAS in the sample.

(e) *Test Compound.* The quantity of test compound which will supply 10 mg of organic carbon should be calculated. If the compound is readily soluble in water, a solution containing 10 mg per ml of organic carbon in distilled water should be prepared.

(f) *Procedure.* (1) Test compound at 10 mg of organic carbon per liter of basal medium should be added in a test flask.

(2) LAS at 10 mg of organic carbon per liter of basal medium should be added in a control flask.

(3) One flask containing basal medium and containing no added test material or LAS should be selected and appropriately labeled as a blank flask.

(4) Each flask should be innoculated with 1 ml of mixed microbial inoculum per liter of basal medium.

(5) The flasks should be placed on a reciprocating shaker or gyratory shaker operating at sufficient speed to assure good aeration (dissolved oxygen 2.0 mg/l or higher) but not so fast as to cause spilling or wetting of the plugs, and then incubated in dark at $22 \pm 3°$ C.

(6) Adaptation: Adaptive transfers should be made at 48 to 72 hour intervals with the final adaptive transfer on the 10th day following the start of the adaptation period. On each transfer day, 1 ml of the 48- to 72-hour culture should be transferred into each liter of fresh basal medium, basal medium plus test compound, and basal medium plus reference compound. Transfers should be from test compound flask to test compound flask, from blank to blank, and from control to control.

(7) Growth of cultures within each flask is indicated by an increasingly hazy or cloudy appearance in the liquid medium and by the deposit of cellular matter on the upper walls of the flasks. If the medium stays clear in the test flask, the test substance might be toxic or inhibitory. In this case, the test procedures should be repeated with the substance at a lower

concentration. If the control flask also remains unclouded, defective inoculum is possible.

(8) Test Period: On the thirteenth day following the initial inoculation, 72 hours after the final adaptive transfer, another transfer into test flasks should be made. The procedure is the same as for the adaptive transfer, except for duplicate preparations for blanks, controls, and test flasks, and two flasks with basal medium plus test compound but without inoculum. The contents of the latter two flasks should be kept sterile throughout the test period.

(9) Following transfers, the first samples should be taken from each flask, the height of the liquid marked with a water-insoluble marker, and the samples incubated as before.

(10) Samples should be taken over a period of 21 days on 0, 1, 2, 3, 4, 7, 10, 15, and 21 or by some other schedule appropriate to the rate of degradation for the test chemical. Only the necessary volume of culture medium necessary for each DOC analysis should be withdrawn, mixing well to dissolve or resuspend material adhering to flask walls before sampling. Evaporation losses should be made up between samplings with distilled water. Following each removal of sample, the liquid level should be marked before returning the flask to the shaker. Unless analyses are run immediately, samples should be preserved by acidifying to pH 2 or less with concentrated H_2SO_4, HCl, or HNO_3 (note: choice of acid may depend on compatibility with the organic carbon apparatus). Preservation can be done before or after filtration.

(11) Within three hours of sampling, each sample should be filtered through a 0.2 μm pore-size membrane until there is sufficient filtrate for the organic carbon analysis. Filters should be pretreated in boiling water for one hour and then stored (for up to one week) at room-temperature in distilled water.

(12) A total organic carbon analysis (TOC) of the filtrate measures dissolved organic carbon (DOC). TOC instruments suitable for the DOC determinations in the range of 1 to 10 mg C/l include products from Beckman Instruments, Dohrman-Envirotech, Ionics Corp., and Oceanography International. Each sample should be analyzed at least twice and the average value reported as mg C/l, using the format shown below.

(13) If suitable methods are available, analysis of the test compound can be used to follow disappearance of that compound. A complete description of the test method and results should be provided.

The % removal at time t should be calculated for each replicate and for each sampling time using the equation

$$\%\ \text{Removal}_t = \frac{[(C_0-B_0) - (C_t-B_t)]\ 100}{(C_0-B_0)}$$

where % removal_t = % removal at time t

C_0 = Concentration of organic carbon in test or reference flask at time 0

B_0 = Concentration of organic carbon in blank flask at time 0

C_t = Concentration of organic carbon in test or reference flask at time t

B_t = organic carbon in blank flask at time t.

The % removal values should be averaged for each pair of duplicate flasks and plotted against the time in days.

(g) *Calculations and Information to be Recorded.* (1) The Shake Flask Test data sheet (Table 1) is offered as the preferred format for recording test data, with DOC values expressed as mg C/l to the nearest whole number.

(2) The % removal values vs. time should be calculated and recorded.

(3) A plot of % removal vs. time should be recorded.

(4) Analytical data (for example, if there are analyses of the parent compound) with a description of the method and results should be recorded.

Table 1. Data Sheet for Shake Flask Test: DOC Analysis

Test Material: Starting Date:_____

Name:_____ Type of Carbon Analyzer:

Initial Conc. (mg/l):_____ _____

Reference Material

Name:_____

Initial Conc. (mg/l):_____

Culture medium	Replicate	Average mg C/l on day								
		0	1	2	3	4	7	10	15	21
Basal medium with inoculum only	1									
	2									
Basal medium with test cmpd + inoculum	1									
	2									
Basal medium with reference cmpd + inoculum	1									
	2									
Basal medium with test cmpd only	1									
	2									

A-4.52 *Activated Sludge Test.* (a) *Test Description.* The activated sludge test is based on the semicontinuous activated sludge test of the Soap and Detergent Association (1965) and on the static activated sludge method of Zahn and Wellens (1974) and Zahn and Huber (1975).

Following a period of adaptation to synthetic sewage and gradually increasing concentrations of test compound, an activated sludge should be exposed to a mineral salts medium plus test compound in a well aerated chamber for up to 20 days. Biodegradation is followed by comparing dissolved organic carbon (DOC) at the start with DOC in the mixed liquor on the test day.

(b) *Aeration Chambers.* Polyethylene or glass 3 to 4 l. percolators (or other suitable vessels with cone shaped lower ends) should be fitted with an

aeration stone (gas diffuser) secured at the inside of the narrow end of each percolator. Each percolator should be suspended vertically in a ring or by a bale holder and clean (oil-free) compressed air should be provided to the aeration stone by means of tubing passed through the small, cone shaped end of the percolator. The small end of the percolator should be sealed to make it watertight and 2 l. of water should be added to each chamber. The outside of each chamber should be marked indelibly at the 2 l. liquid level and the water discarded.

(c) *Activated Sludge.* Activated sludge should be collected from a sewage treatment plant that principally treats domestic wastes. The suspended solids concentration (APHA, 1975) should be determined and then adjusted to 2500 mg/l by adding tap water or by decanting supernatant, as appropriate.

(d) *Synthetic Sewage Stock Solution.*

Glucose	30.0 g
Nutrient broth	20.0 g
Dipotassium hydrogen phosphate	13.0 g
Ammonium sulfate	2.5 g

Tap water should be added to make 1 liter and the ingredients dissolved by heating just below the boiling point. The solution should be stored in a refrigerator at less than 7°C. The stock solution should be discarded if evidence of biological growth appears.

(e) *Mineral Nutrient Solution.* (1) 109.55 g $CaCl_2 \cdot 6H_2O$ should be dissolved in distilled water and made up to 1 liter.

(2) 123.25 g of $MgSO_4 \cdot 7H_2O$ should be dissolved in distilled water and made up to 1 liter.

(3) Nutrient solution: 38.5 g of NH_4Cl, and 9.0 g of $NaH_2PO_4 \cdot H_2O$ should be dissolved in distilled water and made up to 1 liter.

(4) Mineral nutrient solution: For each liter of Mineral Nutrient Solution, 4.8 ml of $CaCl_2$ solution, 0.6 ml of $MgSO_4$ solution, and 2.5 ml of the Nutrient Solution should be used and diluted to 1 liter with distilled water.

(f) *Acclimation Procedures.* (1) 200 ml of the solids-adjusted activated sludge should be placed into each chamber and aerated at a rate of about 500 ml/minute.

(2) A daily "fill and draw" routine should be done as follows:

(i) Chamber walls should be scraped and/or brushed down to remove accumulated solids and to return them to the aerated liquor.

(ii) The mixed liquor suspended solids (MLSS) concentration should be determined and, if necessary, sufficient mixed liquor removed to maintain MLSS at 2000 to 3000 mg/l.

(iii) Aeration should be stopped and the solids allowed to settle for 30 minutes.

(iv) The upper 1500 ml of supernatant should be discarded, leaving 500 ml of settled sludge and liquor in the aeration chamber.

(v) Aeration should then be resumed.

(vi) 15 ml of synthetic sewage stock should be added to each chamber, and the chambers then filled to the 2000 ml mark with tap water.

(vii) The pH should be checked daily and maintained in the range 6.5 to 8.0 by adding H_2SO_4 or NaOH solution, as necessary.

(viii) The dissolved oxygen concentration of the mixed liquor should be checked daily and, if necessary, the air flow should be increased to maintain at least 2.0 mg/l D.O. in each unit.

(3) With each run, one blank unit should be maintained which is fed synthetic sewage stock only.

(4) With each run, one reference compound unit should be maintained which is fed 15 ml of synthetic sewage stock daily plus diethylene glycol, according to the following schedule:

Acclimation day	Diethylene glycol, mg/unit	Total mg as organic carbon
0	0	0
1, 2, 3	88	40
4, 5, 6	177	80
7, 8, 9	265	120
10, 11	354	160
12, and daily to end of acclimation period	442	200

(5) With each run, four test compound units should be maintained which are fed 15 ml of synthetic sewage stock daily plus test compound, using the following schedule:

Acclimation day	Test compound, total mg as organic C	
	Units 1 and 2	Units 3 and 4
0	0	0
1, 2, 3	20	40
4, 5, 6	40	80
7, 8, 9	60	120
10, 11	80	160
12, and daily to end of acclimation period	100	200

Test compound should be added on the basis of its calculated organic carbon content. The test compound may be added as a solution in water (if sufficiently soluble) or as aliquots weighed to the nearest 0.1 mg.

(6) All units should be maintained at 22°±3°C.

(7) Beginning on the fourteenth day, following the sludge settling step, a portion of the supernatant should be retained for DOC analysis. Simulated influent samples should be prepared as follows:

(i) Blank influent: 1.5 ml synthetic sewage feed + distilled water to 200 ml.

(ii) Referencce unit influent: 1.5 ml synthetic sewage feed + 44.2 mg diethylene glycol + distilled water to 200 ml.

(iii) Test units influents: 1.5 ml synthetic sewage feed + test compound at 10 or 20 mg as organic carbon + distilled water to 200 ml.

Each sample of supernatant and simulated influent is then filtered through a 0.2 μm pore-size membrane until there is sufficient filtrate for the organic carbon analysis. If filtration proves difficult, the sludge solids can be partially removed by centrifugation prior to filtration. The filtrate should be preserved for later analysis, by acidification to pH 2 or less with concentrated H_2SO_4, HCl, or HNO_3 (the choice of acid depends on compatibility with the specific TOC apparatus).

(8) This procedure should be continued for at least three days (days 14 through 16 of the acclimation period) with the same feeding schedule and daily sampling and analysis.

(9) The daily % removal of dissolved organic carbon (DOC) should then be calculated:

$$\% \text{ Removal} = \frac{I - S}{I} 100$$

where

I=DOC of simulated influent
S=DOC of supernatant sample

(10) The daily data should be plotted and the acclimation program continued until the DOC removal efficiency remains at a high (>70%) and consistent (±10%) level for 3 consecutive days or until the thirtieth day of the acclimation period. If the acclimation period continues for 30 days without achieving high DOC removal consistently for 3 consecutive days, the test should be concluded at that time.

(g) *Test Procedures.* (1) Following the three-day period of consistently high DOC removal in the acclimation procedures, and approximately 24 hours after the last addition of synthetic sewage, sufficient mixed liquor should be discarded to reduce the suspended solids to 1000 mg in each unit (500 mg/l). Aeration should be stopped and the remaining solids allowed to settle for 30 minutes. All of the clarified supernatant above the settled sludge solids should be discarded. Aeration should then be resumed and material added to each chamber, as follows:

(i) Blank unit: Mineral nutrient solution to the 2000 ml mark.

(ii) Reference compound unit: 442 mg of diethylene glycol plus mineral nutrient solution to the 2000 ml mark.

(iii) Low concentration test units: 100 mg of test compound, as organic carbon, plus mineral nutrient solution to the 2000 ml mark.

(iv) High concentration test units: 200 mg of test compound, as organic carbon, plus mineral nutrient solution to the 2000 ml mark.

(2) Test unit temperatures should be maintained at 22±3°C, pH at 6.5 to 8.0, and D.O. at 2.0 mg/l or higher. Aeration should be continued throughout the test period.

(3) Losses from evaporation and sampling should be made up before each sampling with nutrient solution (the solution given in (e)(5)(C)) diluted 1:500 with distilled water. A total volume of 2000 ml should be maintained to the conclusion of the run. The volume removed for sampling is taken into account in the evaluative calculations.

(4) The removal of dissolved organic matter should be determined by DOC measurements at regular intervals. Samples from the test, reference, and blank units should be filtered through pre-washed 0.2 μm pore-size membranes. If filtration proves difficult, the sludge can be partially removed by centrifugation prior to filtration. The filtrate should be preserved by acidification for later analysis.

(5) The first samples should be taken 30 minutes after beginning the test to detect adsorption of test compound on the activated sludge. Subsequent samples should be taken at 3, 6, and 24 hours and on days 2, 3, 4, 6, 8, 10, 13, 16, and 19 at approximately the same time of day.

(6) The percentage removal of DOC is given by:

$$\% \text{ removal} = \frac{[DOC_A-(DOC_T-DOC_K)]\ 100}{DOC_A}$$

where

DOC_A = initial DOC value based on amount of compound added. DOC_A will be 100 mg/l for diethylene glycol and either 50 or 100 mg/l for test compound at the start of the test. These values should be corrected for sample volumes as described below.

DOC_T = value at time of sampling.

DOC_K = value of blank unit sample.

(7) Decline in concentration due to the removal of samples is calculated as in the following example:

A sample of 20 ml. removes 1/100th of the DOC in a total volume of 2000 ml. The initial DOC value (DOC_A) needs to be corrected for this removal, as shown below. This results in DOC_A values that become progressively smaller as the run proceeds.

Time	DOC_A MgC/L	Correction for next DOC_A	Measured DOC - DOC_K mgC/L	% Removal
0.5 hr.	100	$\frac{(80)(20)}{2000} = 0.8$	80	$\frac{(100-80)(100)}{100} = 20$
3 hrs.	100-0.8 = 99.2	$\frac{(70)(20)}{2000} = 0.7$	70	$\frac{(99.2-70)100}{99.2} = 29$
6 hrs.	99.2-.7 = 98.5	$\frac{(45)(20)}{2000} = 0.45$	45	$\frac{(98.5-45)100}{98.5} = 54$
24 hrs.	98.5-.45 = 98.05	$\frac{(32)(20)}{2000} = 0.32$	32	$\frac{(98.0-32)100}{98.0} = 67$

NOTE: Correction for next DOC_A = measured DOC × $\frac{\text{sample volume}}{\text{total volume}}$

(8) The test period should last no more than 19 days. If 90% removal is attained before the end of 19 days and is confirmed by a second analysis on the following day, the trial can end. The test may also be discontinued if the percent removal values do not appear likely to reach 90% and remain consistent (±10%) for 3 or more consecutive days.

(9) If suitable analytical methods are available, the disappearance of the test compound may be followed.

(h) *Calculations and Information to be Recorded.* (1) Daily % removal during acclimation period, including calculations and a plot of % removal vs. time should be recorded.

(2) The data for each sample during the test period should be recorded.

(3) The % removal values for each unit at each sampling time during the test period, including calculations, should be recorded.

(4) The average % removal for each duplicate at each sampling time should be recorded.

(5) A plot of % removal vs time should be recorded.

(6) Any additional analytical data (for example, analyses of the parent compound), with a description of the test method and the results should be recorded.

A-4.53 *Methane and Carbon Dioxide Production in Anaerobic Digestion.* (a) *Test Description.* The anaerobic digestion test is based on the procedures of Nottingham and Hungate (1969). Approaches that might be followed, and acceptable methods for conducting anaerobic aquatic biodegration studies are cited below.

An anaerobic sludge is obtained from a municipal treatment plant. Portions of this stock culture are transferred to stoppered containers and allowed to equilibrate. Selected containers receive the test material. The amount of gas produced in each container is measured periodically and the collected gas is analyzed for its methane and carbon dioxide content. Methane production in units receiving test material compared to controls (no supplement) provides information on the biodegradeability of the substrate under anaerobic conditions.

(b) *Procedures.* An anaerobic digestor sludge should be obtained from a municipal waste treatment plant and portions placed in each of several closed containers. The containers may be butyl-rubber stoppered test tubes (16 × 150 mm) as described by Nottingham and Hungate (1969) or larger tubes or flasks (100 to 500 ml capacity). The 16 × 150 mm test tubes should receive about 5 ml of sludge. Other types of containers should receive sludge at about 25 ml of sludge per 100 ml of capacity. Prior to closing the units, air should be displaced with O_2-free carbon dioxide gas. If small tubes are used, evolved gas may be collected and analyzed using the procedures of Nottingham and Hungate (1969). If larger vessels and larger quantities of sludge are employed, it may be necessry to trap evolved gases in a displacement type gasometer, such as that shown by Mueller, et al. (1959) and by Weng and Jeris (1976).

The units should be maintained at 35°C to 37°C. Evolved gas should be collected and the volume produced should be measured daily, until the daily gas production volume becomes relatively constant (±10%) for 3 or more consecutive days.

When the sludge-containing units reach equilibrium with respect to gas production, test material should be added according to the following schedule:

Unit No.	Test material (mg/l)
1, 2	0
3, 4	10
5, 6	20
7, 8	40
9, 10	100
11, 12	200

Until reference compounds are identified, another pair of units, numbered 13 and 14, should be provided with a single dose of the NYG medium described by Nottingham and Hungate (1969) at the rate of 1 ml of NYG medium per 100 ml of sludge.

The cultures should be incubated at 35° to 37°C for at least 28 days. Gas production is measured periodically (as convenient) and the evolved gas is analyzed for methane and carbon dioxide (Nottingham and Hungate, 1974). At the end of the test period, 5 *N* HCl is added to each unit at the rate of 10 ml of HCl per 100 ml of sludge. This will convert bicarbonate to CO_2. The evolved gas should be measured and analyzed for methane and CO_2 to end the test. If a suitable method is readily available for analysis of the test compound, the vessel contents should be analyzed at the completion of the test period.

As discussed by Nottingham and Hungate (1969), it is possible to calculate the theoretical maximum production of methane and carbon dioxide derived from anaerobic degradation of the test compound. Excess gas production in the units receiving test compound, as compared to units which were unfed, may be related to anaerobic digestion of the test compound. The excess methane and CO_2 (as mg of carbon) produced during the test period is compared to the total theoretical maximum and the percent of theoretical production then calculated as shown.

$$\% \text{ of theoretical} = \frac{(G_T - G_M)\ 100}{G_T}$$

where

G_T=total mg of organic carbon in sample
G_M=mg of carbon in excess CH_4+CO_2 (excess compared to units 1 and 2)

(c) *Calculations and Information Which Should be Recorded*

(1) The source of sewage sludge and date of sampling should be recorded.

(2) Total gas production for each culture unit at each measurement time should be recorded.

(3) Methane and carbon dioxide content of the gas at each measurement time from each unit should be recorded.

(4) The calculated % of theoretical CH_4 and CO_2 production for the test period at each substrate concentration should be recorded.

(5) Repression (if any) of gas production in the presence of test material should be recorded.

A-4.54 *Carbon Dioxide Evolution.*—(a) *Test Description.* The procedures suggested for use in screening for ultimate biodegradability potential (CO_2 evolution and BOD measurement) are based on the method described by Sturm (1973) and on the standard biochemical oxygen demand (BOD) test (APHA, 1975). Those investigators who conduct biodegradability tests should choose to run only one of these methods. The carbon dioxide evolution method is preferred over the long-term BOD test.

(b) *Acclimated culture.* The CO_2 evolution test should be preceded by a thirteen-day acclimation period, using the same materials and procedures described for the shake flask method, with the following exceptions:

(1) The reference compound should be glucose instead of LAS. A concentration of 10 mg C/l of glucose should be made by using 25 mg/l in the basal medium.

(2) Organisms may be acclimated to more than one test compound simultaneously by setting up separate shake flasks for each compound.

(3) On the thirteenth day, equal volumes (500 ml or more) from each shake flask culture should be combined, including the blank and control flasks, to form a composite inoculum culture.

(c) *Procedures.* The procedures should be as follows:

(1) A reaction unit of about 4 l. volume should be set up and air trains attached as described by Sturm (1973), with air passing through a rotameter, a drying tube (Ascarite), and a CO_2 check (100 ml of 0.05 N $Ba(OH)_2$) before entering the carboy and passing through three CO_2 absorbers (100 ml of 0.05 N $Ba(OH)_2$ in each) arranged in a series, after leaving the reaction chamber.

(2) 250 ml of composite inoculum and 2750 ml of mineral salts medium (basal medium without the yeast) should be placed into each reaction unit. A separate unit and air train apparatus should be set up for a blank, for the reference compound (glucose), and for each test compound concentration.

Each unit containing inoculum and mineral salts medium should be aerated with CO_2-free air for 24 hours prior to adding test compound.

(3) Each test material should be added to its respective reaction unit to concentrations of 5 and 10 mg C/l.

(4) The mixtures should be incubated in the dark at 22±3° C.

(5) CO_2-free air should be bubbled through each setup and the effluent air from each unit passed through three CO_2 absorbers (in series), each containing 100 ml of 0.05 N $Ba(OH)_2$. Periodically, the CO_2 absorber most proximal to the carboy should be removed for titration. The remaining two absorbers should each be moved one place closer to the carboy and a new absorber unit placed at the distal end. The air flow should be low enough that entrainment of aerosols is avoided and evaporation of trapping solution is prevented.

(6) The proximal absorber should be removed for titration on days 1, 2, 3, 4, 5, 8, 10, 15, 21 and 28 following addition of the test compound. The analysis schedule may be varied by the investigator as deemed appropriate to match the degradation rate of rapidly degraded or relatively resistant material.

(7) CO_2 produced during the test and trapped as barium carbonate should be quantitated by titrating the $Ba(OH)_2$ remaining in solution to the phenolphthalein end point with 0.1 N HCl. It generally is more convenient to titrate a portion of the absorber solution, e.g. 25 ml, and to calculate CO_2 production from that data. The procedures and data for each of the absorbers—blank, control, and test units should be recorded.

(8) Calculations: The calculations should be as follows: The ml of 0.1 N HCl required to titrate the residual $Ba(OH)_2$ in the absorber from the blank unit should be recorded. The ml of 0.1 N HCl required to titrate the residual $Ba(OH)_2$ in the absorber from the test unit should be recorded. The test value should be subtracted from the blank value to determine the ml of 0.1 N HCl required to titrate the $Ba(OH)_2$ not converted to $BaCO_3$ by CO_2 derived from the test or control compound. This value multiplied by the normality of the HCl equals the milliequivalents (meq) of HCl required and also is equal to the meq of CO_2 produced from the substrate. Note that 1 meq of CO_2 = the gram molecular weight of CO_2 divided by 2000=44/2000=0.022g=22mg.

As an example:

0.1 N HCl to titrate 25 ml of blank unit = 6.3 ml
0.1 N HCl to titrate 25 ml of test unit = 5.4 ml
difference = 0.9 ml

(0.9 ml) (0.1 N)=0.09 meq HCl=0.09 meq CO_2 produced
0.09 meq CO_2 =(0.09) (22)=1.98 mg CO_2 in 25 ml of absorber solution
(1.98) (4)=7.9 mg CO_2 in 100 ml of absorber solution

Overall equation:

$$\text{mg of } CO_2 \text{ produced from substrate} = \frac{(T_b - T_s)\ (0.1)\ (22)\ (100)}{V} = \frac{(T_b - T_s)\ (220)}{V}$$

where,

T_b = ml of 0.1 N HCl required to titrate aliquot from blank absorber

T_s = ml of 0.1 N HCl required to titrate aliquot from test compound absorber

V = ml of aliquot used in titration

CO_2 production should be calculated for each time period preceding the collection of each absorber unit.

The maximum theoretical CO_2 production from each test or control unit (due to substrate oxidation) is 55 mg for test compound at 5 mg C/l and 110 mg for test compound at 10 mg C/l and for reference compound.

The accumulative % of theoretical CO_2 actually produced at each sampling time should be calculated. For example:

$$\%\ \text{theoretical } CO_2 = \frac{\Sigma[CO_2]_1^n\ (100)}{110} = \frac{\Sigma[CO_2]_1^n}{1.1}$$

where $\Sigma[CO_2]_1^n$ = the sum of CO_2 production values from the absorber samples taken on day 1 though day n.

(9) The % of theoretical CO_2 vs. time in days should be plotted.

(d) *Calculations and Information Which Should be Recorded.*

(1) The calculated CO_2 production for each time period and the accumulative total CO_2 production at each sampling time should be recorded, for each experimental unit.

(2) The calculated % theoretical CO_2 production values should be recorded for each unit at each sampling time.

(3) A plot of % theoretical CO_2 production vs. time should be developed and recorded.

A-4.55 *Biochemical Oxygen Demand (BOD).* (a) *Test Description.* See A-4.5(g)(1).

(b) *Acclimated Culture.* The procedures for obtaining acclimated culture should be the same as for the CO_2 evolution test.

(c) *Dilution Water.* The dilution water should be the mineral salts medium used for the CO_2 evolution test. The dilution water should be prepared one day or more before use and stored in the dark at 20°C in a loosely stoppered container until just before use. One to two hours before use, the dilution water should be inoculated with 0.1 to 0.5 ml of mixed microbial inoculum per liter of dilution water and the water gently aerated for 15 minutes with oil-free compressed air (an aquarium pump is convenient for this purpose).

(d) *Procedures.* (1) If the test compound is sufficiently soluble in water, a solution containing 0.3 mg per ml of test compound should be prepared. If the pH of this solution is less than 6.5 or greater than 8.0, the pH should be adjusted to the 6.5 to 8.0 range, as appropriate, with H_2SO_4 or NaOH solution.

(2) A standard reference solution should be prepared containing 150 mg/l each of reagent grade glucose and glutamic acid that have been dried at 103°C for one hour before weighing.

(3) Eighteen 300 ml BOD bottles should be filled about half full with inoculated dilution water, and additions of standard reference solution or test compound made as shown below.

BOD bottle No.	Organic compound additions
1, 2	None.
3, 4	5 ml of standard reference solution.
5, 6	10 ml of standard reference solution.
7, 8	0.3 mg of test compound.
9, 10	0.6 mg of test compound.
11, 12	1.2 mg of test compound.
13, 14	2.4 mg of test compound.
15, 16	4.8 mg of test compound.
17, 18	None.

The test compound may be added as a water solution if sufficiently soluble or as aliquots weighed to the nearest 0.1 mg. Each bottle should be filled to the top with inoculated dilution water and the dissolved oxygen immediately determined. During this and all subsequent D.O. determinations, the D.O. in the bottles should be measured in numerical order with two blanks first and the other two blanks always last.

(4) Each bottle should be stoppered and incubated in the dark at 20°C.

(5) The dissolved oxygen in each bottle should be measured on days 1, 2, 3, 4, 5, 8, 10, 15, 21, and 28, following addition of test compound. The contents of each bottle should be topped off with distilled water, as necessary, to maintain a water seal. To minimize evaporation of the water seal during incubation, the bottle tops may be covered with small caps or pliable film.

(6) The analysis schedule may be varied by the investigator as deemed appropriate to match the biodegradation rate of rapidly degraded or relatively recalcitrant material.

(7) The D.O. readings for each day should be subtracted from the D.O. readings for the same bottle on day zero. This gives the D.O. depletion value. From this the average depletion value for the blanks should be subtracted to obtain a "depletion less blank" value. This figure should then be multiplied by the inverse of the dilution factor to obtain the BOD value. Those cases where there is a residual D.O. of at least 1 mg/1 and a depletion of at least 2 mg/1 should be considered the most reliable values. An example is shown:

Bottle No.	Bottle No. and dilution	D.O. values			Depletion less avg. blank	Con'n factor	BOD
		Day 0	Day 4	Depletion			
1	Blank	7.6	7.3	0.3			
2	Blank	7.7	7.2	0.5			
7	1/10 ⁶	7.7	7.2	0.5	0.1	10^6	
8	1/10 ⁶	7.7	7.2	0.5	0.1	10^6	
9	3/10 ⁶	7.6	6.1	1.5	1.1	5×10^5	
10	3/10 ⁶	7.7	6.1	1.6	1.2	5×10^5	
11	1/10 ⁵	7.6	5.1	2.5	2.1	2.5×10^5	5.3×10^5
12	1/10 ⁵	7.5	4.9	2.6	2.2	2.5×10^5	5.5×10^5
13	3/10 ⁵	7.5	2.8	4.7	4.3	12.5×10^4	5.4×10^5
14	3/10 ⁵	7.5	2.9	4.6	4.2	12.5×10^4	5.3×10^5
15	1/10 ⁴	7.4	0.8			62.5×10^3	
16	1/10 ⁴	7.3	0.8			62.5×10^3	
17	Blank	7.7	7.3	0.4			
18	Blank	7.8	7.4	0.4			

The more reliable calculated BOD values should be averaged to provide, for this example, a four-day BOD for the organic compound of 538,000 mg of O_2 per 10^6 mg of compound.

(8) Given the molecular structure of the test compound, it is possible to calculate the amount of oxygen required to completely oxidize the molecule to carbon dioxide, water, and other inorganic molecules. Comparing this value with the BOD results for any given day provides a measure of the % of the theoretical O_2 uptake:

$$\%\ \text{of theoretical} = \frac{[BOD_T - BOD_M]\ 100}{BOD_T}$$

where

BOD_T = theoretical O_2 requirement for complete oxidation

BOD_M = measured BOD.

(e) *Calculations and Information Which Should be Recorded*

(1) The calculated BOD values for each day, complete with the data on which the calculations were based should be recorded.

(2) The average BOD values for test and reference samples each test day should be recorded.

(3) The calculated % of theoretical O_2 uptake for each day for the test compound should be recorded.

(4) A plot % of theoretical O_2 uptake vs time should be developed and recorded.

A-4.56 *References.*

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B. Health Effects Testing

There has been considerably more research and experience in the field of health effects testing than in environmental effects or chemical fate testing. As a result, reasonably well-established tests exist for acute toxic effects, subchronic effects, mutagenicity, teratogenicity, reproductive effects, oncogenicity, and chronic toxicity.

The National Academy of Sciences (1977) has issued a recent report on methods for evaluating toxic health effects,* as has the World Health Organization** (1978) and the Food Safety Council (1978).*** Also, EPA has proposed health effects testing guidelines under section 3 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).**** In general, EPA regards any health effects test performed as described in any of these sources to be appropriate for TSCA section 5 premanufacture notification purposes. With respect to EPA's preferred testing methods, this spring the Agency will propose test standards under section 4 of TSCA for a number of health effects tests. These test methods will be incorporated, as appropriate, into section 5 testing guidelines. In preparing proposed section 5 guidelines for health effects testing, EPA will consider previous comments on the section 4 test standards as well as the FIFRA guidelines. However, the Agency welcomes comments at this time on the construction of and the applicability of these references guidelines for the development of health effects information related to section 5 assessment needs.

C. Ecological Effects Tests

C-1 *Discussion of Ecological Effects.* Releases of hazardous chemical substances into the environment during manufacturing, processing, distribution, use, or disposal, whether accidental or planned, can have an adverse impact on both natural and man-modified ecosystems and their components. The societal costs may include degradation of the environment, losses in sport and commercial fisheries, shellfish, populations and wildlife resources, losses in agriculture, losses in tourism and property values, and other adverse impacts.

The chemical fate and health effects tests described in sections A and B above will often provide data helpful in evaluating the potential ecological effects of chemical substances. However, such effects are exerted differently on diverse life forms, as well as at various levels of biological organization.[1] Testing for such effects requires the selection of indicators (i.e., indicative parameters) that provide for wide taxonomic representation and include a range of biological processes.

Ideally, testing at levels of ecological organization above the individual species would provide information more directly related to ecological consequences of the release of a hazardous chemical. However, the development and standardization of such tests is difficult due to the complexity of the species interactions that characterize ecosystems. A major thrust for the future therefore will be the development of test methods that address interactions such as those which occur between predator and prey, among competitors for habitat or food, and between disease and host organisms. As methods such as microcosm studies and other laboratory model systems are developed, they may help to address these ecological testing needs.

Laboratory testing below the level of the organism is also potentially useful. It is generally rapid and readily amenable to standardization, but most such testing has not yet been shown to be useable for ecological impact assessment. Since many cellular and subcellular functions are common to a wide range or organisms, they have the potential of being applicable to many sets of ecological circumstances. Therefore, there are a number of subcellular tests which are also being considered for future inclusion in testing and assessment strategies.

However, most ecological effects tests currently in use employ single species test populations of vertebrates, invertebrates or plants. Individual species represent an intermediate level of biological organization between subcellular functions and community/ecosystem interactions. Many single species tests are considered to be state-of-the-art and have correlated well with actual ecological effects of chemicals. As a consequence, the ecological effects tests included in these guidelines are all single species tests. As subcellular and ecosystem level tests are developed and validated they will be added to the guidelines.

The tests included in this section are those to which a manufacturer may refer when a case-by-case analysis indicates that preliminary data on ecological effects will be needed for assessment of a chemical substance. These guidelines do not provide criteria for making such a chemical-specific decision, nor for selection of specific tests when ecological testing is warranted, nor for the design of advanced tests (which must also be determined on a case-by-case basis). The specification of standardized test methods always rests on the current state of the methodology. Standardized tests

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*Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council, 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Prepared for the Consumer Product Safety Commission. National Academy of Sciences: Washington, D.C.

**Principles and Methods for Evaluating the Toxicity of Chemicals. Part I. World Health Organization. Geneva, 1978.

***Proposed System for Food Safety Assessment. Report of the Scientific Committee of the Food Safety Council (1978). Food Safety Council, 221 Teachers Building, Columbia, Maryland, 21044.

****Proposed Guidelines for Registering Pesticides in the U.S.; Hazard Evaluation: Humans and Domestic Animals. Federal Register Volume 43, No. 163, Tuesday, August 22, 1978, Part II, pp. 37336-37403.

[1] Subcellular, cellular, organismal, population, community and ecosystem levels.

are therefore subject to modification, revision, or replacement as improved methods and assessment strategies become available. This section will address some of the uncertainties inherent in preliminary testing for ecological effects, some of the areas in which future improvement might occur, and the general importance of ecological effects testing.

Generally, the following criteria were used to select tests for recommendation in these guidelines:

- The test is in standard use, generally acceptable to the scientific community, or considered to represent a state-of-the-art methodology.
- The test results are significant and useful for risk assessment.
- The test applies to a wide range of chemical substances or categories of chemical substances.
- The test is cost-effective in terms of personnel, time and facilities.
- The test is adequately sensitive for detection of the subject effect.

Confidence in extrapolation from simple tests to ecologically-significant impacts depends not only on the appropriate kinds of tests but also on selection of appropriate organisms to be used in those tests. Organisms useful for assessment testing should have characteristics such as the following:

- The organism is representative of an ecologically important group (in terms of taxonomy, trophic level, or realized niche).
- The organism occupies a position within a food chain leading to man or other important species.
- The organism is widely available, is amenable to laboratory testing, easily maintained, and genetically stable so uniform populations can be tested.
- There is adequate background data on the organism (i.e., its physiology, genetics, taxonomy, and role in the natural environment are well understood) so data from these tests can be adequately interpreted in terms of actual environmental impacts.

C-2 *General Provisions*—C-2.1 *Quality Assurance.* EPA is currently drafting Good Laboratory Practices (GLP) under section 4(b) of TSCA. When these become available, they will provide detailed guidance on proper procedures for laboratory management and the conduct of tests for ecological effects.

C-2.2 *General References.* The Pesticide Guidelines proposed under Part 162, Chapter 1, Title 40 of the Code of Federal Regulations (Pesticide Guidelines Subpart E, Hazard Evaluation: Wildlife and Aquatic Organisms. FEDERAL REGISTER 43(132): 29724-29727) are recommended as the main reference for any ecological effects tests not addressed in the following discussions. As test standards are developed and published under section 4(b) of TSCA, and by the Interagency Regulatory Liaison Group (IRLG), they will also serve as generally recommended tests for use under section 5 of TSCA. Other suggested references are noted under the individual tests contained in these guidelines.

C-2.3 *Reporting on Test Substance.* (a) Data obtained by tests conducted with the technical grade product to be marketed by the manufacturer will be most relevant for assessment purposes, unless that is not the most probable formulation to which organisms will be exposed or unless indicated differently in a specific test method listed below. If the technical grade product is not tested, an explanation of the reason for the substitute would help validate the data for assessment. If possible, a description of the differences between the substance that was used and the market product, including a description of the physical state and chemical nature and how these may affect biological characteristics should be given.

(b) Test results will be more consistent if the lot of the substance tested is the same throughout the duration of the study, and if the sample is stored under conditions that maintain its purity and stability. If a test substance will not be stable for the duration of the study or if, for other reasons, it is not possible to use the same lot throughout the test, subsequent lots of the test substance should be selected that are as nearly identical to the original lot as practicable. Chemical assays should be performed to assure this composition.

C-2.4 *Calculations and Data Which Should be Recorded.* (a) *General.* The data generated by testing are most useful for assessment if submitted in a report format. Such a report should include all information necessary for a generally complete evaluation of the test procedures and results, including information on the facility performing the study, information on the test substance, a description of the test procedures, presentation of the data generated, and a summary and analysis of the test results. The following information is considered valuable for assessment:

(b) *Information on Testing Laboratory/Sponsor.* (1) Name(s) and address(es) of the facility performing the study, and of the sponsor, if different from the testing facility.

(2) Laboratory study number.

(c) *Information on Test Substance.* (1) identification of the test substance, including chemical name, chemical abstract number, sponsor code number, synonyms, and molecular structure.

(2) A qualitative and quantitative determination of chemical composition (including names and quantities of known contaminants and impurities, so far as is technically feasible).

(3) Name of the manufacturer, lot number(s) of test substance, method of synthesis, date(s) when received and storage procedures.

(4) Relevant physical properties of the test substance, such as physical state, pH, stability, melting point, purity and solubility.

(5) Identification and composition of any vehicles or other materials used in testing the substance.

(d) *Description of Test Procedures.* (1) Specification of test methods, including a full description of the experimental design and procedures, reference(s) to the chosen test method, the length of the study, and the dates on which the study began and ended.

(2) Methods of all chemical analyses performed for determining concentrations of test substances, impurities or degradation products, along with the precision and accuracy of the test methods.

(3) Appropriate statistical methods used to summarize experimental data, express trends, and evaluate the significance of differences in data from individual test groups. For example, data averages or means accompanied by standard deviation allow assessment of the variability in the raw data. In addition, standard errors of the means may be used to compare means from different test groups. However, notations of statistically significant differences, along with the confidence level of probability, could substitute for standard errors.

(4) References to statistical methods for analyzing the data, and to published literature used in developing the test protocols, performing the test, making and interpreting observations, and compiling and evaluating the results.

(e) *Description of Organisms.* (1) Community type, species, strain(s) and/or subline(s) used, and rationale for their selection, if different from those recommended by EPA.

(2) Source of organisms, populations, or communities.

(3) Breeding history or breeding type (inbred, hybrid, outbred, as appropriate).

(4) Description of any pretest conditioning or acclimation, including diet and quarantine.

(5) Initial number of each sex, size, and weight in each test group and control group, as appropriate.

(6) Age and condition of each organism, population or community at beginning of study.

(7) Method of randomizing organisms, populations or communities into test groups and control groups.

(f) *Environmental Conditions.* (1) Source of water, its chemical characteristics, and description of any pretreatment, if applicable.

(2) The photoperiod, type of bulb and intensity when light sources are used.

(3) Source, composition, and amount of feed or nutrients made available to organisms.

(4) Temperature and humidity of test environment, if applicable (e.g., humidity is not relevant for aquatic tests).

(5) Description of test chambers including height, width, depth and volume of chamber and contained test environment.

(g) *Preparation of Test Mixture.* Data on preparation of test mixtures which would be useful include name of chemical(s), name of carrier(s), carrier concentration, method(s) of preparation of test mixture, frequency of preparation, physical state, pH, stability and decomposition, storage, and date(s) prepared. If it is known that the test chemical undergoes significant change or loss in the test mixture over the time required for a test, the test mixture should be prepared fresh before each application.

(h) *Exposure.* (1) Dosage or concentration.

(2) Method, route, and frequency of exposure and rationale for selecting each.

(3) Total volume of material (i.e., test substance plus carrier) contained in individual exposures.

(4) The method of randomization used in selecting samples to assay, the assay method used to determine the stability and homogeneity of the test substance in the testing environment, and the results of these assays.

(5) For each exposure level, the mean total amount of test substance administered per organism, population or community.

(6) The reason for selection of the carrier.

(i) *Treatment for infectious disease.* A description of the treatment(s) used to prevent or control infectious diseases if such treatment was undertaken during a test or shortly before a test was begun.

(j) *Observations.* (1) A description of the method(s) of observation.

(2) A description of the frequency of observation, such as when individual organisms are weighed or cultures checked.

(k) *Summary and analysis of test results.* A summary and analysis of the data, and a statement of the conclusions drawn from the analysis. The summary could highlight positive data or observations, and deviations from control data which may be indicative of ecological effects.

C-2.5 *Miscellaneous Considerations.* (a) There are a number of incidental effects which may influence test results; photochemical degradation is one example. Whenever practical, such reactions should be avoided. For example, photochemical degradation can be minimized by shielding reaction mixtures from incident light during the experimental period for animal and bacterial tests. When such reactions are known and cannot be avoided, they should be recorded.

(b) The use of trade names in the text is not intended as an endorsement by EPA, but merely serves as an example of the kind of equipment and facilities that may be used to carry out the tests described.

C-3 *Microbial Effects Tests*—C-3.1 *General.* Microbial activities discussed in these guidelines are associated with three major biogechemical cycles (i.e., carbon, nitrogen, and sulfur cycling) and with decomposition of organic matter. These biogeochemical tests are models which integrate physical components of the environment with biotic processes and indicate not only possible toxic activity, but also effects on the primary mediators of the cycles, the microbes. Data obtained will provide preliminary indications of possible effects of the test chemical on the cycling of elements and nutrients in ecosystems. In addition, the tests will aid in the formation of hypotheses about the ecological effects of chemicals, hypotheses which can be used in the selection of higher level tests when appropriate.

Data on the effects of chemicals on microbial populations and functions can be obtained from laboratory studies employing nonradioisotopic analytical techniques. Studies of effects on microbial functions constitute a more direct approach and are preferred to studies of effects on populations. The activities to be observed are:

- Organic matter (cellulose) decomposition by following CO_2 evolved from organically bound carbon.
- Nitrogen transformations by following the release of organically bound nitrogen (in urea) and the formation of ammonia.
- Sulfur transformation by following the reduction of sulfate to sulfide by *Desulovibrio.*

Two of these tests employ pure cultures of microorganisms for ease of performance and better reproducibility. Additional testing, if suggested by adverse effects near expected environmental concentrations, should employ mixed populations from natural sources. Other functions, such as nitrogen fixation, dehydrogenase activity, invertase activity, nitrification, and pectin or protein degradation, should be considered when additional testing is indicated.

The principal objective of preliminary microbial effects tests is to indicate whether or not the test chemical may cause undesirable changes in microbial populations or in the functions of microbial communities. Such changes might result in a reduction in plant growth or quality through nutritional disturbances, or they might interfere with the natural degradative functions of microorganisms which play a dominant role in transformations of biotic and xenobiotic wastes.

The investigator may choose to substitute more complex procedures, such as those contained in the Pesticides Guidelines (1978), for the methods described here, if the methods employed are commensurate with the intended conditions of chemical use and release to the environment and if the same microbial functions (cellulose degradation, ammonification and sulfate reduction) are investigated.

REFERENCE

Environmental Protection Agency. 1978. Guidelines for registering pesticides in the United States, Office of Pesticides Programs. FEDERAL REGISTER 43 (132):29718-29722.

C-3.2 *Test 1: Cellulose decomposition.* (a) *Objectives.* Cellulose is one of the most abundant organic materials in plants. Dead plant matter, consisting largely of cellulose, is degraded by a number of species of bacteria and fungi. This degradation is accomplished in a stepwise manner going from cellulose to cellobiose to glucose to organic acids and CO_2.

The degradation of cellulose is of particular interest to farmers. The fertility of farmland depends in part on the presence of organisms which degrade dead plant matter. The organisms which decompose the various forms of cellulose are some of the most important microorganisms contributing to humification processes in soils. The inhibition of, or interference with, cellulose degradation by toxic chemicals adversely impacts the recycling of carbon and soil fertility by retarding the breakdown of the vast amounts of cellulose that enter the soil. All chemical substances, except water-insoluble gases, should be considered for this test.

(b) *Rationale.* There are two basic methods available for evaluating cellulose degradation by microorganisms. One method uses variations of soil burial tests in which a cellulose substrate is buried in soil containing a mixed culture that includes cellulolytic microorganisms. The other type employs a liquid culture of cellulolytic microorganisms and measures evolved CO_2. The second is the type proposed here.

One advantage of the liquid culture method over soil burial is time. Typical soil burial tests last for 7 to 8 weeks while liquid culture requires only 7 days. In addition, test compounds in liquid culture are more apt to be in contact with the microorganisms than in soil burial tests because possible soil sorption of test substance is avoided. Pure culture studies in liquid medium also reduce the possibility of lowered toxicity due to biodegradation of the test material. Studies using pure cultures have the two advantages of simpler experimental procedures and greater reproducibility of data.

The major disadvantages of pure cultures are that they are a poor representation of the natural environment and that they fail to allow for synergistic and competing reactions. It could be argued, for example, that inhibition of *Trichoderma longibrachiatum*, specified in this test, should not be taken as evidence that the test compound will inhibit cellulose degradation since other organisms in the soil may be unaffected by the concentration of test material and may degrade cellulose and/or transform the test material. This argument has some validity and only additional testing with mixed cultures in natural soil samples would resolve that question. Tests of that sort, however, are too complex and are not adequately standardized to be justified by the data needs of a preliminary assessment.

(c) *Test Description.* In the proposed test, pure, ball-milled cellulose is added to a mineral salts growth medium as the sole carbon source. This mixture is then innoculated with a known cellulase-producing microorganism and added to an apparatus which allows for growth of the microorganisms and collection of evolved carbon dioxide (CO_2). The quantity of CO_2 produced in the presence of the test substance in comparison with CO_2 production in the absence of the test substance would indicate the effect on microbial decomposition of cellulose.

(d) *Methods*—(1) *Test details.* The cellulase-producing organism which can be used in this test is *Trichoderma longibrachiatum* (ATCC 26921), formerly *T. viride*. Pure stock cultures of the organism are maintained by biweekly subculture on a growth medium for cellulase-producing microbes (Eggins and Pugh 1961). The medium consists of $NaNO_3$, 0.5g; K_2HPO_4, 1.0g; $MgSO_4.7H_2O$, 0.5g; $FeSO_4.7H_2O$, 0.01g; cellulose (ball milled) 12.0g; agar; and distilled water, 1.0 liter, and is modified by the following addition: 0.1g of $CaCl_2$ and 0.5g of $(NH_4)_2SO_4$ per liter.

The apparatus for organism culture and CO_2 collection has been described by Bartha and Pramer (1975). The apparatus allows for semistatic aeration of growing cultures with the cultures receiving CO_2-free air throughout the test. One hundred ml of liquid growth medium (as above but without agar) is despensed aseptically into flasks along with the test compound at one or several concentrations. Testing at five or more concentrations, at least three of which inhibit cellulose decomposition by more than 10% and less than 90% of the control response, supports extrapolation of results to any expected environmental concentration.

Such results are not needed for environmental assessment if the data show that a test chemical is so slightly toxic at reasonably expected environmental concentrations that no detriment to the microbes or to the cellulose degradation process is likely.

For each run of this test, a stock inoculum is prepared by suspending *Trichoderma* spores from the stock culture in distilled water. Stock inoculum for each run should be prepared no more than one hour prior to inoculating flasks. One ml of *Trichoderma* spore inoculum is added to each flask and the flasks are incubated at 22±3°C in darkness. At days 1, 3, and 7, CO_2 evolved by the cultures and collected in the CO_2 absorbant (KOH) is determined titrimetrically. Bartha and Pramer (1965) describe the procedure for introduction and recovery of the KOH from the incubation flask. Appropriate controls consist of flasks containing (1) *Trichoderma* spore inoculum and liquid growth medium and (2) liquid growth medium only (to account for chemogenic CO_2 production). Three replicates of each treatment and each control should provide adequate replication.

(2) *Calculations and Data Which Should be Recorded.* The quantity of CO_2 measured (as mg CO_2) in the presence, and absence, of the test chemical on days 1, 3, and 7 are the data needed for assessment.

(e) *References.*

Bartha, R., and D. Pramer, 1965. Features of a flask and method for measuring the persistence and biological effects of pesticides in soil. *Soil Sci.* 100 (1): 6870.

Eggins, H. O. W., and Pugh, G. J. F. 1961. Isolation of cellulose-decomposing fungi from soil. *Nature*, Lond. 193:9495.

C-3.3 *Nitrogen Transformation.* (a) *Objectives.* Almost all microorganisms, higher plants, and animals require combined nitrogen. In addition, the nitrogen cycle is a major biogeochemical cycle. The main aspects of this cycle are the fixation of gaseous nitrogen, the ammonification of organically bound nitrogen, nitrification, and denitrification.

Ammonification is a key initial step in the reintroduction of nitrogen from protein wastes into the soil and is one of the more readily measured reactions of the nitrogen cycle. As soon as an organism dies and its organic waste returns to the soil, biological decomposition begins and fixed nitrogen is released. The breakdown of proteins and other nitrogen-containing organics in soil and the production of ammonia are the work of widespread and varied microflora. The amino groups are split off to form ammonia in a series of reactions collectively called ammonification. Urea, a waste product found in urine, is also decomposed by numerous microorganisms with the formation of ammonia. This reaction can serve as a convenient assay method for ammonification activity. There is a strong correlation between an organism's ability to degrade urea and its capacity to degrade protein.

The ammonification test presented below is potentially applicable to all chemical substances except water-insoluble gases. Information from such testing would be used to assess the likelihood that the test chemical interferes with the normal conversion of organically-bound nitrogen into ammonia.

(b) *Rationale.* Consideration of at least some aspects of the nitrogen cycle are essential for assessing the effects of substances on microorganisms. An easily measured feature of the nitrogen cycle is the oxidation of nitrite to nitrate by *Nitrobacter* bacteria (Winely and San Clemente, 1970). This focuses on a part of the nitrogen cycle less important than the critical step involving the conversion of organic nitrogen into ammonia.

The preferred method uses urea as a readily obtainable, reproducible nitrogenous organic compound. Some investigators have used pieces of liver or kidney tissue, vegetable meal, dried blood, and casein hydrolysate as nitrogen sources, but these substances are not standardizable. Percolation techniques may also be used for the same purposes in the study of soluble proteins, peptones, polypeptides, and amino acids in solution (Greenwood and Lees, 1956). Methodology using fertile soil and urea and following the evolution of ammonia nitrogen is relatively simple to conduct and will provide meaningful results. Urea is a suitable nitrogen source not only because it is readily available and relatively easy to handle, but in addition, the ability to degrade urea has been associated with general proteolytic capabilities.

Nonsymbiotic nitrogen fixation, nitrogen mineralization and ammonification usually show no response to applications of toxic chemicals such as herbicides (R. Rubinstein et al. 1975). Nitrification is one of the most sensitive conversions in the soil. Nitrification may be inhibited by concentrations of chemicals not inhibiting other impor-

tant biochemical reactions so there are reasons for choosing nitrification as the process which would give results most useful for assessment. One of the arguments against this, as noted earlier, is that ammonification is a more vital part of the nitrogen cycle than nitrification. In addition, the temporary inhibition of nitrate formation is often beneficial in that it slows down the loss of nitrogen from the root areas of plants by leaching and denitrification. Lastly, nitrification by *Nitrobacter* may be too sensitive to inhibition by chemicals in laboratory studies and might give results not representative of natural circumstances and environments. Although inhibition of nitrifying activity could lead to ammonium ion accumulation and serious problems such as root damage, ammonification is considered the more appropriate process to be examined as a first step in looking for effects on the nitrogen cycle.

(c) *Test Description.* The response measured in this test is the ammonification of urea nitrogen by a microbial assemblage in fresh soil or sediment. Although this test uses urea as the nitrogen-containing compound to be mineralized, it may be modified to allow estimation of the mineralization of other N-containing materials.

(d) *Methods*—(1) *Test Details.* Urea is added to sieved soil or sediment (1 mm fraction) along with the test chemical. Testing at five or more concentrations, at least three of which inhibit ammonification activity by more than 10% and less than 90% of the control response, supports extrapolation of results to any expected environmental concentration. Such data are not necessary for assessment if the test substance produces no toxic or inhibitory effects, as determined by this test, at or near reasonably expected environmental concentrations and if no detriment to the ammonification process is likely.

The preferred procedure for this test has been described by Curl and Rodiguez-Kabana (1972). Other procedures which are suitable have been described by Porter (1965), McGarity and Myers (1967), and Greaves et al. (1978). Appropriate controls are: (1) incubation vessels containing soil or sediment only, and (2) incubation vessels containing soil or sediment, and nitrogenous amendment only. Incubation should be at 22±3° C for 2 to 3 weeks. Three replicates of each treatment and control should provide adequate replication. Suitable methods for the analysis for ammonia nitrogen may be found in "Methods for Chemical Analysis of Water and Wastes" (1974).

(2) *Calculations and Data to be Recorded.* Ammonia production for each test unit at each sampling time and the cumulative ammonia production for each test unit over the complete duration of the test is needed for assessment. Ammonia production should be reported as milligrams of ammonia nitrogen (mg NH_3N) per 100 grams of oven dry soil.

(e) *References.*

Curl, E. A., and R. Rodringuez-Kabana. 1972. *Research Methods in Weed Science* R. E. Wilkinson (ed.) Southern Weed Science Society, pp. 178–197.

Environmental Protection Agency. 1975. *Methods for Chemical Analysis of Water and Wastes.* EPA-625/6-74-003.

Greaves, M. P., et al., 1978. Methods of analysis for determining the effects of herbicides on soil microorganisms and their activities. *Technical Report,* Agriculture Research Council, Weed Research Organization. No. 45, p. 55.

Greenwood, D. J. and Lees, H. 1956. Studies on the decomposition of amino acids in soil. I. A preliminary survey of techniques. *Plant Soil* 7:253.

McGarity, J. W., and M. G. Myers. 1967. A survey of urease activity in soils of Northern New South Wales. *Plant Soil* 27:217–238.

Porter, L. K. 1965. Enzymes. *In* C. A. Black (ed.), *Methods of Soil Analysis* (pp. 1536–1549). American Society of Agronomy, Madison.

Rubinstein, R., et al. 1975. *Test Methods for Assessing the Effects of Chemicals on Plants* (p. 3-6). Prepared for Office of Toxic Substances. U.S. Environmental Protection Agency. EPA 560/5-75-008.

Winely, C. L. and C. L. San Clemente. 1970. Effects of pesticides on nitrite oxidation by *Nitrobacter agilis. Appl. Microbiol.* 19:214–19.

C-3.4 *Test 3: Sulfur Transformation (Sulfate Reduction).* (a) *Objectives.* The sulfur cycle is one of the major biogeochemical cycles. Sulfur is found in soil, waters, and the atmosphere in its most reduced form as a result of volcanic action and the decomposition of organic matter. Free sulfur occurs in some soils, and the oxidized form, sulfate, occurs in all soils. Both sulfur and hydrogen sulfide (H_2S) are oxidized by combustion and by microorganisms to sulfates which are used by plants. Sulfur is essential to all living organisms as a part of sulfur-containing amino acids. It is released from organic compounds (plant and animal wastes) by anaerobic decomposition, as H_2S, in its most reduced state.

Sulfate-reducing bacteria are widely distributed in nature where anoxic conditions exist—as in sewage, sediments, muds, and in bovine rumina. Two groups of bacteria are able to reduce sulfate to H_2S (Frobisher et al. 1974). The best known of these are the *Desulfovibrio* organisms. These organisms have been thoroughly studied because they are responsible for serious odor and corrosion problems associated with sulfate reduction. However, these problems tend to obscure the necessary and beneficial role played by *Desulfovibrio* in the sulfur cycle.

Data on sulfur transformation (sulfate reduction) is potentially applicable to the assessment of all chemical substances except water insoluble gases (i.e., those with solubility in water of less than 1 mg/1 at 30°C). If it can be demonstrated that the minimum inhibitory concentration is greater than the highest concentration tested and also, that the highest concentration tested is significantly higher than reasonably expected environmental concentrations, it may be assumed that no detriment to the sulfate reduction process is likely.

(b) *Rationale. Desulfovibrio* was chosen as the test organism because it plays an important role in the sulfur cycle and because it functions under conditions of low oxidation-reduction potential (anaerobic conditions) and will provide some indication of potentially adverse effects under such conditions.

Standard methods for the determination of sulfate-reducer activity are described in Standard Methods (APHA, 1975) and by the American Society for Testing and Materials (1978). The Standard Methods procedure 2a.1 (p. 1001) was selected as being reproducible and relatively simple and as adequate for the screening test.

(c) *Test Description.* The test for inhibitory effects on sulfate reducer activity is a modified time-contact evaluation with the organism and the test compound in contact with each other for a selected period of time. Following the period of contact between test compound and *Desulfovibrio* suspension, the organisms are transferred into a sulfate-reducing medium.

(d) *Methods*—(1) *Test details.* The sulfate-reducing organism *Desulfovibrio desulfuricans* strain Hildenborough (NCIB 8303) should be used in this test. The organism can be obtained as freeze dried material from cultures which have been grown at 30°C (Saleh, *et al.,* 1964). Stock cultures of the organism should be maintained by weekly subculture in a growth medium for the heterotrophic growth of sulfate-reducers (Adams and Postgate, 1961). The medium consists of: K_2HPO_4, 0.5g; NH_4Cl, 1.0g; Na_2SO_4, 1.0g; $CaCl_2.6H_2O$, 0.1g; $MgSO_4.7H_2O$, 2.0g; sodium lactate (70 percent solution), 3.5g; yeast extract (spray dried), 1.0g; $FeSO_4.7H_2O$, 0.002g; sodium thioglycollate, 0.1g; ferrous ammonium sulfate 0.5g; water, to make 1.0 litre, final pH 7.5.

Fifteen ml of a 4- to 6-day *Desulfovibrio* culture should be placed in 1485 ml of fresh sulfate-reducer medium. This stock culture is inverted once to distribute the microbial inoculum. The stock culture should then be dispensed, in 99 ml aliquots, into eleven screw cap-type milk dilution bottles and the bottles numbered one through eleven. One ml aloquots from bottles 1

and 2 should then be added to two screw cap culture tubes (20 x 125 mm), the culture tubes filled to capacity with sulfate-reducer medium, sealed and numbered 1 and 2. As described below, chlorine solution or test chemical should then be added to bottles 3 through 11 to provide the indicated final concentrations:

Aliqout bottle No.	Addition	Final conc. of test material (mg/l)
1, 2	None	0
3	Chlorine	1
4	do	10
5	do	50
6	Test Chemical	1
7	do	10
8	do	50
9	do	100
10	do	500
11	do	1000

A suitable chlorine stock solution should be prepared from hypochlorite solution, containing 30,000 to 50,000 mg/l chlorine equivalent (Standard Methods, 1976, p. 346).

Following addition of chlorine or test chemical, each bottle should be capped and the contents mixed. The bottles and tubes should then be maintained at 22±3°C in the dark. At the end of 3 and 24 hours, the contents of each bottle should be mixed by inverting, then 1 ml aliquots from each dilution bottle should be transferred into screw cap culture tubes filled with sulfate-reducer medium. The tubes should then be capped and numbered to correspond to the bottle numbers and labeled to indicate elapsed incubation time. The inoculated tubes should be incubated in the dark at 30°C for 21 days. The procedure for observing the presence of sulfate-reducing bacteria is found in Method 2a.1 of Standard Methods (1976, p. 1001). If growth of sulfate-reducing bacteria does not occur in the control tubes, the entire test should be repeated with a fresh source of inoculum. If sulfate-reducer growth occurs in the controls, but does not occur in any of the test chemical treatment tubes, the test should be repeated using concentrations of the test chemical of 1.0, 0.1, 0.01, and 0.001 mg/l.

(2) *Calculations and Data Which Should be Recorded.* A detailed description of the test procedures should be provided including the preparation and handling of the test compound and the contenst of each bottle and tube. In addition, culture tubes which display evidence of sulfate-reducing bacteria (blackening of the medium) should be noted along with the day on which that evidence was first observed. The minimum inhibitory concentration of test substance should be recorded as greater than the highest test chemical concentration at which growth is observed.

(e) *References.*

Adams, M. E., and Postgate, J. R. 1960. On sporulation in sulphate-reducing bacteria. *J. Gen. Microbiol. 24:291-24.*

American Public Health Association. 1975. *Standard Methods for the Examination of Water and Waste Water* (14th Edition).

American Society for Testing and Materials. 1978. *Annual Book of Standards.* Part 31. Sulfate-reducing bacterial in water and water-formed deposits. ASTM. Philadelphia, PA. pp. 955-959.

Forbisher, M., R. D. Hinsdill, K. T. Crabtree, and C. R. Goodheart. 1974. Pages 674-676 *Fundamentals of Microbiology,* 9th ed. W. B. Saunders Co., Philadelphia.

Saleh, A. M., et. al. 1964. The effect of inhibitors on sulphate-reducing bacteria: A compilation. *J. Appl. Bacteriol.* 27:281293.

C-4 *Plant Effects Tests.* C-4.1 *General.* All organisms require energy to perform vital functions and use radiant energy of sunlight as the ultimate source of energy. Green plants use this energy directly through the process of photosynthesis when suitable inorganic carbon and nutrients are present. The sun's energy is converted into chemical energy which is stored in plants in the form of sugars, starches and other organic chemicals to be used by the plants themselves or by other organisms as energy sources. Photosynthesis is the source of virtually all atmospheric oxygen. Plants also synthesize vitamins, amino acids, and other metabolically active compounds essential to many organisms. In this context, the maintenance of the biosphere depends upon the normal functioning of green plants.

Data from the tests in this section are expected to provide preliminary indications of the effects of chemical substances on the following groups of plants: Blue-green algae, diatoms, green algae, monocotyledons, and dicotyledons.

Blue-green algae are one of two groups of organisms capable of converting atmospheric nitrogen into forms which can be utilized by all living organisms. Diatoms and unicellular green algae are responsible for most of the world's photosynthesis and are the primary food energy base for most organisms inhabiting aquatic environments. They are, therefore, necessary for all aquatic life and for human food taken from fresh water and marine environments (e.g., fish).

Monocotyledons and dicotyledons are the two major groups of terrestrial flowering plants. The most highly evolved and widely distributed plants, they are the source of most human food and fiber, as well as shelter and important medicinals. These groups of plants are recognized as major converters of radiant energy to chemical energy in terrestrial environments.

General criteria for selecting specific organisms for preliminary assessment data are given above in the Discussion of Ecological Effects Tests (section C-1). In addition, plants were considered for their importance as a direct source of food for humans, or for animals consumed by humans. While no battery of test organisms meet all criteria fully, we have selected organisms which, on the basis of the available scientific literature and expert advice, come closest to fulfilling as many criteria as possible.

Other groups, possibly important to ecosystems, were not included. Among these are red algae, brown algae, dinoflagellates, mosses, ferns and gymnosperms. Representatives of these groups are not included in these testing recommendations either because suitable tests are not available, or because these groups of plants are considered secondary in importance to the maintenance of environmental quality, or because they fail to adequately meet one or more of the other criteria.

C-4.2 *Test 1: Algal Inhibition.* (a) *Objectives.* Testing for inhibition or stimulation of the growth of algae indicates the extent to which a test chemical can affect primary producers in lakes, streams, estuaries and oceans. It can also generally indicate phytotoxicity or plant growth stimulation.

Substances which drastically inhibit growth at or near concentrations expected in the environment may reduce aquatic productivity. However, even those substances which inhibit algal growth only partially, or which stimulate growth, at or near concentrations expected in the environment might shift relative algal populations so that undesirable species could increase. In the latter case, it may be important for assessment of certain chemicals to include tests of the relative sensitivity of the major groups of algae: green algae, blue-green algae, and diatoms. If diatoms or green algae grow less in the presence of the chemical than blue-green algae, for example, a bloom of the less desirable blue-green species could develop. Further testing beyond these guidelines could be needed to test that hypothesis.

Substances which have no effect on algal growth at concentrations within several orders of magnitude of the levels expected in the environment would not be expected to harm the environment through effects on algae. Further testing would probably add little additional value for the assessment process.

(b) *Rationale.* Algae were selected to represent aquatic primary productivity because they constitute the major mechanism for fixation of energy in most aquatic locations. Techniques for culturing and measuring them are simpler and less expensive than for larger

plants or for attached organisms (e.g., periphyton).

The parameters recommended to measure potential effects on algae are inhibition of dry weight increase and changes in cell size. There are other potential effects which are not recommended. Lethality, for example, is commonly measured for other organisms, but it is difficult to determine for microscopic organisms and for nonmotile organisms. Inhibition of photosynthesis and/or respiration could be measured, but the balance between photosynthesis and respiration is accumulated as growth. In addition, growth represents the product which is important in the food chain and is therefore more directly relevant to assessment.

An alternative to measurement of dry weight that can be used is measurement by electronic particle counter (Miller, et al., 1978). This method is acceptable for assessment if accompanied by a calibration curve and conversion of data into dry weight equivalents. Dry weight equivalence is needed for comparing all data in assessment.

Uncertainties in using these data in assessment center upon whether the selected species are adequate indicators of the potential for stimulation or toxicity to non-selected algal species, whether benefits of algae to the food chain can be accurately predicted from changes in dry weight and cell size, and whether there are significant interactive effects (such as competition) which are affected at lower concentrations of the chemical substance than any individual species. Inadequacies of species selection result from having a poor data base on toxicity to algae, so relative sensitivity is not well known. The lack of indicators is also due to having no representatives of some major groups such as red algae, brown algae, and dinoflagellates.

Food and particle size are two factors most likely to affect the consumption of phytoplankton in food chains, although taste, texture and nutritional quality may sometimes prove significant. These are relatively unknown areas for planktivorous organisms, and no simple methodologies exist for their evaluation. Competition among algae also limits extrapolation from laboratory tests to field conditions. These areas, therefore, should receive major emphasis in future test development.

(c) *Test Description.* The inhibition or stimulation of algae by chemical substances is determined by inoculating an appropriate growth medium amended with various concentrations of test chemical. When this test is performed, the organisms are separately incubated under conditions favorable for growth for a stanard period of time. Algal growth occuring in the presence of the chemical compared to growth occuring in its absence would indicate the degree of inhibition or stimulation. In addition to observations on growth, observations on the potential for nitrogen fixation for *Anabaena flos-aquae* would be useful for assessment.

(d) *Methods*—(1) *Test details. Anabaena flos-aquae, Skeletonema costatum, Navicula seminulum* var. *hustedtii,* and/or *Selenastrum capricornutum* are grown separately under conditions favorable to the growth of each. When this test is performed, *A. flos-aquae* and *S. capricornutum* should be grown in a suboptimum freshwater medium at 24°C for 7 days (EPA, 1971). Artificial seawater with Na_2SiO_3 should be used as the medium for *S. costatum* (EPA, 1974, p. 10). For *N. seminulum* var. *hustedtii,* the standard medium should have 20 mg of Si/1 added as $Na_2SiO_3.5H_2O$ (Payne and Hall, 1977). Other cultural conditions for *N. seminulum* follow those given above for *A. flos-aquae* and *S. capricornutum* (EPA, 1971). The experimental design and procedures should determine the effects, if any, due to changes in inorganic carbon when the test chemical causes pH shifts. The light intensities given in the references should be used. For toxicity testing, a medium not containing chelating agents is desirable but no EDTA-free medium exists that is as widely accepted as these two media. For *S. costatum,* 24°C is a suitable temperature. The test details of Sikka and Butler (1977) should be used. Hattori (1962) describes the medium and culture conditions for testing the potential for nitrogen fixation.

Growth should be measured as dry weight increase (or by a method calibrated with dry weight increase) and by an indication of cell size. If an automatic particle counter is used, adequate disruption of cell clumps and filaments is necessary for valid results; this is particularly important for the filamentous alga, *A. flos-aquae* (Shiroyama, et al, 1975).

If measurement is by any technique other than dry weight determination, a calibration curve is useful.

The effect on nitrogen fixation in *A. flos-aquae* as measured by reduction of acetylene (Hardy, et al., 1973) contributes to an understanding of potential impact on the nitrogen cycle.

Testing at five or more concentrations, at least three of which inhibit growth by more than 20% and less than 95% of normal growth, and with at least one no effect concentration, permits extrapolation of results to inhibitions at any expected environmental concentration. These concentrations should be spread over the range as evenly as possible. Data at lower concentrations permit conclusions about the possibility of stimulation. More data are not useful for assessment if a substance is non-stimulatory and nontoxic at reasonably expected environmental concentrations.

(2) *Calculations and Data Which Should be Recorded.* The dry weight gain of the test algae obtained in the untreated controls should be included, along with gain in the various test concentrations. The 95% confidence limits should be calculated for each growth response. For each response, any changes in cell size should be included. For *Anabena flos-aquae,* changes in acetylene reduction will serve as an indicator of nitrogen fixation.

(e) *References.*

Eutrophication and Lake Restoration Branch. 1974. *Marine Algal Assay Procedure: Bottle Test.* Environmental Protection Agency, Corvallis, OR. 43 pp.

Hardy, R.W.F., et al. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Bio. Biochem.* 5:47-81.

Hattori, A. 1962. Light induced reduction of nitrite and hydroxylamine in a bluegreen alga, *Anabaena cylindrica. Plant Cell Physiol.* 3:355-369.

Miller, W. E., et al. 1978. *The Selenastrum capricornutum Printz Algal Assay Bottle Test.* Environmental Protection Agency, Corvallis, OR. EPA-600/9-78-018, 126 pp.

National Eutrophication Research Program. 1971. *Algal Assay Procedure: Bottle Test.* Environmental Protection Agency, Corvallis, OR. 82 pp.

Payne, A. G., and R. H. Hall. 1977. A method for measuring algal toxicity and its application to the safety assessment of new chemicals. *Proceedings of the Second Symposium of Algal Toxicity.* Cleveland, OH.

Shiroyama, T., et al. Comparison of the algal growth responses of *Selenastrum capricornutum* Printz and *Anabaena flos-aquea* (Lyngb.) De Brebisson in waters collected from Shagawa Lake, Mn. *Proceedings: Biostimulation and Nutrient Assessment Workshop,* September 10-12, 1975, University of Utah, Logan, PPWG, 168-1, pp. 127-148.

Sikka, H. C., and G. L. Butler. 1977. *Effects of Selected Waste Water Chlorination Products and Captan on Marine Algae.* U.S. EPA 600/3-77-029.

C-4.3 *Test 2: Lemna Inhibition.* (a) *Objectives.* Testing for inhibition of *Lemna* (duckweed) growth can serve as an indicator of the extent to which a chemical substance can interfere with aquatic higher plants. Substances which significantly inhibit the growth of *Lemna* at or near concentrations expected in the environment may be presumed hazardous to this group of organisms. Chemicals which inhibit *Lemna* growth only partially at or near concentrations expected in the environment may shift relative populations of macrophytes and algal species. Therefore, it may be important to include a comparison of the relative sensitivity of *Lemna* and algae. If *Lemna* is considerably less sensitive to a given test chemical than algae, an

extensive growth of macrophytes is a possibility. If data indicate the possibility of severe effects on macrophytes or excessive dominance of macrophytes over algae, more information would be needed. For example, testing the relative sensitivity of beneficial plants (pondweeds, saltmarsh grass) compared to undesirable plants (water hyacinth, watermilfoil) would reduce assessment uncertainties.

Substances which have no effect on *Lemna* at concentrations within several orders of magnitude of the levels expected in the environment, and to which other aquatic organisms (e.g. fish, invertebrates) are probably relatively insensitive, are considered safe for aquatic exposure.

(b) *Rationale. Lemna* was selected to represent aquatic macrophytes because it is well known genetically and physiologically and is one of the few aquatic macrophytes used extensively in culture. It is easy to culture and tests can be performed at less expense than would be possible with larger plants.

Aquatic macrophytes possess many of the developmental/structural characteristics unique to higher plants. Roots are particularly important in this regard because they are frequently more sensitive than other plant parts, and their inclusion among the measurements made on *Lemna* serves as an important indicator of potential toxicity. Unfortunately, roots may also be especially sensitive to such environmental variables as nutrients and temperature. For that reason, and because foliage may also be sensitive, it is important to hold cultural conditions constant during testing. The fact that *Lemna* is not completely typical of higher plants introduces an uncertainty into its use as a test organism. Further research is needed to ascertain that *Lemna* is an adequate indicator, or an alternative aquatic macrophyte should be found.

Of the two general approaches to culturing *Lemna*, the most commonly used is static (batch) culture in which nutrients and test substance are present at the beginning of the experiment and decline as the plant takes them up. A less commonly used technique is a flow-through system in which nutrients and test chemical are continuously replenished during the test (Walbridge, 1977). The static system is considered acceptable, though the flow-through system may be preferable because it maintains constant concentrations of test chemical and nutrients throughout the test.

Whichever system is used, response can be measured several ways. Since roots are generally most sensitive, a measure of their growth often indicates maximum sensitivity of the species. However, roots are less useful in assessment than measures more directly related to productivity and standing crop of plants in the ecosystem. For these two purposes, dry weight and frond number are commonly used. For preliminary assessment, data from either of these latter measurements are suitable. For a more complete hazard evaluation both sets of data would be useful.

There are uncertainties in the counting of fronds which could be avoided by measuring frond area as an indicator of size. Area is suitable for most assessment purposes, and could be included in addition to number of fronds.

The major uncertainty in the use of *Lemna* data in assessment lies in extrapolation to a wide variety of aquatic plants. No single species can fully represent the diverse life forms and habitats of plants which grow in water.

(c) *Test Description.* To determine inhibition of *Lemna minor* or *Lemna gibba* (duckweed) by a chemical, fronds should be placed on an appropriate growth medium amended with several concentrations of chemical and incubated under conditions favorable for growth for a standard period of time. The increase in fronds and dry weight should be measured, and other indications of phytotoxicity occurring in the presence of the chemical compared to that occurring in untreated controls observed.

(d) *Methods*—(1) *Test Details.* Data suitable for assessment can be obtained by growing *Lemna minor* or *L. gibba* on nutrient medium under simulated natural daylight (3000 lux) at 24° for seven days. Growth should be measured as an increase in frond number and root length, with dry weight determined at the end of each experiment (Hillman, 1959, 1961) and observations on necrosis and other effects made. Testing at five or more concentrations of test chemical with at least three inhibiting growth by more than 10% and less than 90% of normal and with at least one no-effect concentration permits extrapolation of results to most expected environmental concentrations. These concentrations should be spread over the range of partial inhibition as evenly as possible. Such data are not useful for assessment if a chemical is so slightly toxic that, at reasonably expected environmental concentrations, no detriment is conceivable.

(2) *Calculations and Data Which Should be Recorded.* Growth of untreated controls, growth with various concentrations of the test chemical, inhibition (or stimulation) with the test chemical compared to the untreated controls, and the mean 50 percent inhibitory concentration should be recorded. The 95% confidence limits and observations of abnormal growth or development should also be included. For each quantified parameter, frond numbers, root length and dry weight are the data useful in assessment. Sublethal effects, such as necrosis, discoloration, or altered morphology, should be recorded whenever observed.

(e) *References.*

Hillman, W. S. 1959. Experimental control of flowering in *Lemna*, II. Some effects of medium composition, chelating agents and high temperatures on flowering of *L. perpusilla* 6746. *Amer. J. Bot.* 45: 489–495.

Hillman, W. S. 1961. The *Lemnaceae*, or duckweeds. A review of the descriptive and experimental literature. *Botan. Review* 27: 221–287.

Walbridge, C. T. 1977. A Flow Through Testing Procedure with Duckweed (*Lemna minor* L.) EPA-600/3-77-108, 20 pp.

C-4.4 *Test 3: Seed Germination and Early Growth.* (a) *Objectives.* Assessment of potential ecological effects of phytotoxic chemicals needs to include an evaluation of possible impacts on terrestrial plants. While all terrestrial plants are of interest in assessment, angiosperms (flowering plants) are of particular concern since they constitute the dominant vegetation in many parts of the United States and are also the source of all major food crops.

These tests are designed to reveal possible adverse effects of chemicals on critical early stages in the life cycles of angiosperms. Chemical inhibition of seed germination or early seedling growth at concentrations which approach expected environmental levels would indicate a potential hazard. To assess further any chemical which causes a significant reduction in germination or seedling growth in any of the test species, additional data should include exposure data and:

(1) Additional seed germination and early seedling growth tests using other species of the same genus, related genera, and unrelated genera to determine if the effect is species specific.

(2) Observations of effects of the chemical over the entire life cycle of the affected species and of additional annual species to show whether the effect is restricted to germination and early stages of seedling growth.

If abnormalities are noted in other growth parameters (e.g., chlorosis, necrosis, abnormal leaf or stem shape), testing directed toward any oberved abnormalities would improve assessment.

Lack of inhibitory effects of a chemical in these preliminary tests does not necessarily mean that the chemical is without potential for causing damage to terrestrial plants, since the tests cover over a few species and only a small portion of their life cycles. If other tests in these Guidelines demonstate that a chemical is

biologically active or subject to high environmental release, more testing of potential effects on reproduction, such as flowering or seed formation, may be needed.

(b) *Rationale.* Chemical inhibition of any of the major physiological processes of higher plants (e.g., photosynthesis, respiration, mineral nutrient uptake, translocation) may interfere with normal growth and development. This, in turn, may cause reproductive failure, disruption of food webs, or decreased crop production. In some cases, chemical inhibition of higher plant growth may decrease vegetational ground cover, causing loss of topsoil through erosion, which, in turn, could cause further decreases in plant growth.

Results of seed germination and early seedling growth tests contribute to assessment because these are critical and easily measured stages in the growth of flowering plants (angiosperms). We recommend the six selected species because they are all either human foods or feeds for domesticated food animals, and represent plant families of ecological and/or economic significance. While more rapid procedures measure specific physiological processes, such as respiration and photosynthesis, we refrain from recommending these because of uncertainties in extrapolating from physiological processed to ecological effects.

Ideally, test results would permit an evaluation of effects of chemicals over the entire life cycle of the test organism. Restricting the test to the early stages of development cannot reveal the possible effects of chemicals on, for example, flower induction and subsequent stages leading to successful sexual reproduction. These processes are essential for plants to perform essential ecological functions and, in many cases, to produce food. Tests for effects of chemicals on these critical life cycle stages are not yet available as standardized methods.

Because early growth is rapid and depends upon a high level of metabolic activity, such growth could be singularly vulnerable to chemical inhibition. Additionally, the cuticle and other structures which protect plants by impeding penetration of chemicals are less well developed in young seedlings than in more mature plants, thus rendering early stages of growth most vulnerable to general inhibition.

(c) *Test Description.* These tests measure effects of chemicals on seed germination and on early stages of growth in seedlings of flowering plants. Percent germination of seeds, shoot height, shoot and root dry weight, and visible abnormalities are measured. Effects of chemicals on seed germination are measured after direct exposure of nongerminated seeds. Effects on seedling growth are measured following exposure of seedlings after seed germination.

All chemicals should be tested by the routes of exposure considered by the submitter as likely to be significant in the environment. If a major route of exposure will be through the roots, then the test chemical should be added to the nutrient medium with which plants are irrigated. Foliar application may be by dusting, or spraying with an appropriate carrier if necessary. If a carrier is used, a control should be included consisting of carrier without test chemical. Chemicals which are gases at environmental temperatures, or which volatize readily at environmental temperatures, are tested for their effects on early seedling growth by fumigation procedures, outlined in Rubenstein et al. (1974).

(d) *Procedures*—(1) *Test Details.* The following are recommended as the preferred species:

MONOCOTYLEDONS

Oats—*Avena sativa* L. 'Clintford'
Ryegrass—*Lolium perenne* L. 'Manhattan'
Corn—*Zea mays* L. 'Butter and sugar'

DICOTYLEDONS

Cucumber—*Cucumus sativus* L. 'Marketeer'
Bean—*Phaseolus vulgaris* L. 'Pinto'
Tomato—*Lycopersicum esculentum* Mill. 'Rutgers'

Pending completion by EPA of the development and validation of other seed germination and seedling development test protocols, the procedures used will be those in Rubenstein et al. (1975), pages 3-35 through 3-46 with the following modifications:

(A) Vermiculite for seedling growth tests should be prewashed with nutrient solution to minimize adsorption of the test chemical.

(B) Fresh weights need not be recorded.

(C) Seeds that have not been treated with pesticides or other chemicals should be used.

(D) For seedling growth tests, seeds should be germinated on sheets of high purity filter paper.

(E) For seedling growth, ED_{50} should be estimated for 50 per cent growth inhibition rather than for lethality.

(F) Seedlings showing any signs of bacterial or fungal infection, or of infestation by insects, during the three week culture period before treatment should be removed.

(G) Where fumigation is required to evaluate the chemical, procedures in Rubenstein et al. (1975), Section 3.14, pages 3-121 through 3-124, and section 1.3.4 should be used.

(2) *Calculations and Data Which Should be Recorded.* Measurements on seed germination, seedling height, and shoot and root dry weight with 95% confidence limits are useful for assessment. All observations of foliar lesions, chlorosis, necrosis, or growth abnormalities should be recorded. Also the results of germination and growth in the presence of test chemical should be calculated as a percentage of untreated controls, including the 95% confidence limits. For fumigation tests, the presence of lesions serves as an indicator of the potential for toxicity. The rationale for selection of test concentrations should be included.

(e) *References.*

Rubenstein, R., et al. 1975. *Test Methods for Assessing the Effects of Chemicals on Plants.* EPA 560/5-75-008.

C-5 *Animal Effects Tests.* C-5.1 *General.* The potential of a chemical to produce adverse ecological effects can be indicated by the results of preliminary testing of "representative" animals. Preliminary tests and test organisms should be selected on the basis of taxonomic, ecological, toxicological, and chemical exposure criteria. Test schemes should reflect those ecological hazards that a specific chemical substance may cause. Test responses—death, reproductive and/or behavioral dysfunction, and impairment of growth and development—are important factors for hazard assessment. Such responses indicate that the need for subsequent testing for effects at levels of ecological organization above the population level, i.e., the community or ecosystem levels.

The tests recommended below represent the current state of methodology for preliminary assessment of ecological hazard. They will be subject to modification or replacement as test methods and assessment strategies are further developed. Additional tests, both general and specific, are available and their use is suggested if preliminary testing indicates a more detailed assessment is necessary.

C-5.2 *Test 1: Acute Toxicity to Aquatic Invertebrates.* (a) *Objectives.* Acute toxicity of a chemical substance to invertebrates is an important factor in preliminary assessment of impact on ecosystems. Invertebrates have broad ecological roles and show various sensitivities to chemicals. Therefore, recommended tests include a variety of invertebrates from various phyla, habitats and trophic levels.

The physical/chemical characteristics of a chemical substance provide important indications about the most appropriate species to use in testing. For example, species living in or consuming sediment are most appropriate for testing chemicals with high affinities for sediment. On the other hand, planktonic or epifaunal invertebrates are more appropriate for substances expected to be present in the water column.

An invertebrate acute toxicity test is potentially useful in evaluating effects of a chemical substance entering natu-

ral waters in either a dissolved or suspended form. Observations made during an acute invertebrate test also provide guidance for the approximate concentrations of test chemicals to be used in a chronic study. For example, a *Daphnia* acute test could be performed before a chronic *Daphnia* test in order to estimate appropriate test concentrations.

(b) *Rationale.* Of the different methods of measuring acute toxicity of chemical substances to aquatic invertebrates, the one recommended (EPA 1975) is standardized and has been extensively used. This test has provided data on a variety of animals and chemicals on which comparisons can be based during risk assessments. The American Society for Testing and Materials and other organizations are expected to publish updated acute methods in the near future. These may supplement or supersede the test given below.

(c) *Test Description.* An EC_{50} determination is carried out using young or immature aquatic invertebrates which are exposed to a chemical substance for 48 or 96 hours. Several concentrations of the chemical substance are used. Observations are made of the number of animals affected and/or the extent of the effect at prescribed intervals. Data are then analyzed to determine the concentration of the chemical substance that effects 50% of the test animal population during the exposure.

(d) *Methods*—(1) *Test Details.* Tests should be performed on a sensitive life stage of the test organisms according to EPA (1975). Useful background information for the conduct of the test may be found in Standard Methods (1975). A list of species that have been used in acute tests, and the preferred test temperature for each is given in Table 1. The appropriate duration of exposure for each organism is given in the main reference (EPA, 1975).

(2) *Calculations and Data Which Should be Reported.* Results should include 24-, 48-, and 96-hour EC_{50} or LC_{50} values and their 95% confidence limits, and the percent of organisms that died or showed the effect in the control treatment. Useful information would include the pH, concentration of the chemical substance and impurities (as practicable) and the oxygen concentration in the aquaria at 0-, 48-, and 96- hr. Loading (grams of organisms per liter), information on behavorial or physiological abnormalities and unusual occurences affecting test conditions should also be reported.

(e) *References.*

Environmental Protection Agency. 1975. *Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians.* The Committee on Methods for Toxicity Tests with Aquatic Organisms. Ecological Research Series. EPA-660/3-75-009.

Standard Methods for the Examination of Water and Wastewater. 1975. American Public Health Association. (14th ed.) APHA, New York. 762 pp.

TABLE 1.—*List of Species for Acute Tests*

Species	Test temperature (C)*
Marine and estuarine invertebrates:	
Copepods:	
Acartia tonsa	22
Acartia clausi	12
Shrimp: *Penaeus setiferus, P. duorarum, P. aztecus*	22
Grass shrimp: *Palaemonetes pugio, P. intermedius, P. vulgaris*	22, 22
Sand shrimp: *Crangon septemspinosa*	17
Shrimp: *Pandalus jordani, P. danae*	12
Bay shrimp: *Crangon nigricauda*	17
Mysid shrimp: *8Mysidopsis bahia*	22
Blue crab: *Callinectes sapidus*	22
Shore crab: *Hemigrapsus* sp., *Pachygrapsus* sp.	12
Green crab: *Carcinus maenas*	22
Fiddler crab: *Uca* sp	22
Oyster: *Crassostrea virginica, C. gigas.*	22
Polychaetes: *Capitella capitata*	22
Freshwater invertebrates:	
Daphnids: *Daphnia magna, D. pulex, D. pulicaria*	20
Amphipods: *Gammarus lacustris, G. fasciatus,* or G., *pseudolimnaeus*	17, 17
Crayfish: *Oronectes* sp., *Cambarus* sp., *Procambarus* sp., or *Pacifastacus leniusculus*	17, 22, 17
Stoneflies: *Pteronarcys* sp.	12
Mayflies: *Baetis* sp. or *Ephemerella* sp.	17
Mayflies: *Hexagenia limbata* or *H. bilinata*	22
Midges: *Chifonomus* sp.	22
Snails: *Physa integra, P. heterostropha, Amnicola limosa*	22
Planaria: *Dugesia tigrina*	22

* Freshwater amphipods, daphnids, and midge larvae should be cultured and tested at the test temperature. Other invertebrates should be held and tested within 5° C of the temperature of the water from which they were obtained. They should be tested at the recommended test temperature if it is within this range; otherwise they should be tested at the temperature from the series 7, 12, 17, 22, and 27° C that is closest to the recommended test temperature and is within the allowed range.

C-5.3 *Test 2: Acute Toxicity to Fish.*

(a) *Objectives.* Acute toxicity of a chemical substance to fish is an important factor in assessing its potential impact on aquatic ecosystems.

An acute toxicity test on fish is appropriate in preliminary assessment of potential effects from dissolved or suspended chemical substances that are expected to enter natural waters. In addition, acute test results can be used for determining test concentrations for chronic, subchronic and bioconcentration tests for fish.

(b) *Rationale.* EPA's currently preferred methods for acute testing of freshwater and marine fishes (EPA 1975) will be used for this test. The American Society for Testing and Materials and other organizations are expected to publish updated acute test methods in the near future and these may supplement or supersede EPA (1975).

EPA would prefer that manufacturers not conduct acute tests on the goldfish (*Carassius auratus*) although it is listed in EPA (1975), because the goldfish is less sensitive to certain classes of compounds than other species. In addition, fathead minnows (*Pimephales promelas*) should normally not be the only species tested, even though they are a good species for chronic exposure tests, because, like the goldfish, they are less sensitive to certain classes of compounds than other commonly tested species. At certain times of the year, however, other species of proper size and age may not be available, leaving fathead minnows, which are easily reared year-round in the laboratory, as the species of choice.

Rainbow trout (*Salmo gairdneri*) and bluegill sunfish (*Lepomis macrochirus*) are suggested as standard test species. These two fish, one a cold water species and the other a warm water species, have generally been the most sensitive to most previously tested chemical substances. The choice of species may depend upon the geographical area of expected chemical release and available testing facilities.

If salt water exposure is probable, testing a marine species is advised. Even though marine species generally are no more sensitive than fresh water species, toxic effects can be significantly modified by water chemistry.

(c) *Test Description.* An LC_{50} determination is carried out using young fish exposed to a chemical substance for 96 hours. Several concentrations of a chemical substance are used and the number of fish killed are observed at prescribed intervals. Data are analyzed to determine the approximate concentration of chemical substance that will, on the average, decrease the test fish population by 50% in 96 hours.

(d) *Methods*—(1) *Test details.* EPA (1975) gives adequate procedures and examples of acceptable test species. Of the species listed in this reference, only goldfish (*Carassius auratus*) is not recommended. For the sake of uniformity in data, the preferred test species is either rainbow trout (*Salmo gairdneri*) or bluegill sunfish (*Lepomis macrochirus*).

(2) *Calculations and Data Which Should be Recorded.*

Results should include 24-, 48-, and 96-hour LC_{50} values and their 95% confidence limits, the percent of organisms that died in the control treatment, and the loading (grams of organisms per liter) in each treatment. Other useful information would include pH, concentration of test chemical and impurities (as practicable), and oxygen concentration in the aquaria at 0-, 48-, and 96-hours. Observations of behavioral and physiological abnormalities and unusual occurrences af-

fecting test conditions should also be reported.

(e) *References.*

Environmental Protection Agency. 1975. *Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians.* The Committee on Methods for Toxicity Tests with Aquatic Organisms. Ecological Research Series EPA-660/3-75-009.

C-5.4 *Test 3: Quail Dietary Test.* (a) *Objectives.* The objective of a quail dietary test is to give preliminary indication of possible effects of a chemical substance on terrestrial birds. This test is designed to determine the concentration of a substance in food that will be lethal to 50 percent of a test population as well as to observe behavioral, neurological, and physiological effects. The test is appropriate for a chemical which might be found in or on terrestrial bird food.

(b) *Rationale.* The recommended method (EPA, 1975) is the most complete and generally applicable method for performing a quail dietary test. The bobwhite quail (*Colinus virginianus*) is an appropriate test species since it is native to the United States, a desirable sports species, easily and economically reared, widely available, and generally more sensitive to many hazardous chemicals than other common test species.

(c) *Test Description.* An avian five-day LC_{50} is performed by feeding birds food containing a known concentration of test chemical substance for five days. Surviving birds are fed uncontaminated food for another three days. The main purpose of the test is to estimate the concentration of substance that will kill one-half of the birds fed the substance for five days. During both feeding periods, birds are observed for possible sublethal toxic effects.

(d) *Methods*—(1) *Test Details.* The preferred test species is bobwhite quail (*C. virginianus*). A suggested procedure is presented in EPA (1975).

(2) *Calculations and Data Which Should be Recorded.* It is suggested that the calculated LC_{50}, the 95% confidence interval, and methods used be recorded. In cases where the LC_{50} exceeds 5000 ppm, neither calculated median response level nor 95% confidence limits are necessary. Sample size at this dose should be 10 or greater. If any birds die at the lowest dose tested, sequentially lower doses should be given to produce a dose-response series that includes at least one level in which no mortality occurs. The slope of the dose-response relationship should be estimated. However, tabulation of response data is sufficient when the method does not allow calculation of a slope. An estimated nondiscernible effect level would greatly help the assessment process. A description of the nature, time of occurrence, severity, and duration of any observed toxic effect is suggested, including death, behavioral or physiological abnormalities and gross pathological abnormalities. The total volume of material administered and concentrations of chemical substance consumed in the feed during the 5-day exposure period should be recorded.

(e) *References.*

Environmental Protection Agency 1975. Office of Pesticide Programs, FIFRA Registration Guidelines Subpart E, Hazard Evaluation: Wildlife and Aquatic Organisms. FEDERAL REGISTER 40 (123): 26915.

C-5.5 *Terrestrial Mammals.* (a) *Rationale for Omission of Tests.* Wild terrestrial mammals are omitted from the recommended preliminary tests for ecological effects, since EPA believes the acute health effects tests will provide a preliminary estimate of acute hazard to terrestrial mammals. A review of the published comparative toxicology data (Tucker and Crabtree, 1970; Fairchild, 1977; Office of Science and Technology, 1971) supports this assumption, showing that for most compounds, common laboratory species (rats, mice) are equally or more sensitive than mule deer, wild mice, or European ferrets.

Many other responses of wild terrestrial mammals to toxicants (e.g. reproduction) cannot be measured by tests using laboratory mammals. EPA may recommend additional testing for these responses in wild mammals at a future date.

(b) *References.*

Fairchild, E. T. 1977. *Agriculture Chemicals and Pesticides: A subfile of the NIOSH Registry of Toxic Effects of Chemical Substances.* U.S. Department of Health, Education, and Welfare.

Office of Science and Technology. 1971. *Ecological Effects of Pesticides on Non-Target Species.* Executive Office of the President.

Tucker, R. K. and D. Crabtree. 1970. *Handbook of Toxicity of Pesticides to Wildlife.* Bureau of Sport Fisheries and Wildlife. Denver Wildlife Research Center Res. Publ. No. 84.

C-5.6 *Test 5: Daphnia Life Cycle.* (a) *Objectives.* Aquatic invertebrates are the most common food chain links between phytoplankton and desirable species of fish and shellfish. The extent to which chemical substances affect reproduction and growth of aquatic invertebrates is important since healthy stocks of fish and shellfish are dependent on adequate sources of food. This test is applicable to new chemicals that are soluble or suspendable in water, and are predicted to enter natural freshwater systems.

(b) *Rationale.* Few tests have been developed and validated for invertebrates which cover the entire life cycle. A life cycle test is desired for assessment because it would give a better estimate of total hazard than, for example, an acute toxicity test. One of the most widely performed and economical life cycle test uses *Daphnia*, a freshwater zooplankton. *Daphnia* have a planktonic existence, short life cycle, and can be easily cultured. They have been widely used in toxicological testing, and are sensitive to toxicants. Although no invertebrate life cycle tests have been completely standardized, the *Daphnia* life cycle test has been used extensively by many researchers. Some problems exist for determining the best methods for toxicological studies and for culture. However, until better test systems are developed, *Daphnia* life cycle tests will provide the best data for evaluating effects of chemicals on reproduction and longevity of freshwater zooplankton. In addition, when a population of *Daphnia* are healthy and growing, individuals reproduce parthenogenically. This may present some problems in drawing conclusions about organisms which do not reproduce in this manner.

(c) *Test Description.* Reproduction and life-cycle tests on *Daphnia* begin with newborn daphnids, which are exposed to a chemical substance in culture for approximately three or four weeks. Reproductive impact of the chemical substance is evaluated by comparing the number of young produced by the organisms exposed to a chemical substance with the number produced by controls. Chronic lethal effects are evaluated by observing survival of the daphnids initially exposed throughout their life cycle.

(d) *Methods*—(1) *Test details.* Testing conditions can vary, but a satisfactory test should insure that the control *Daphnia* are healthy. Generally, more than 80% of a healthy control *Daphnia* population will survive 28 days and produce more than 100 young each during that period. To be useful, a test should include concentrations of test chemical that show no effect on reproduction as well as levels with a statistically significant effect upon reproduction. These concentrations should be within tenfold of each other.

No experimental design can be recommended, at this time, but the test should meet the standard for longevity and reproduction stated above. The literature shows no clear consensus for a particular food type, for *Daphnia pulex* or *Daphnia magna*, or for flow-through exposure rather than renewal. Published protocols available for *Daphnia* life cycle testing (Standard Methods, 1976; Beisinger, 1975; Winner, 1976) are not standardized or validated with respect to number of animals used, duration of test, water hardness and quality, photoperiod,

food, performance of control animals, temperature, statistical analysis and presentation of data, number of animals per test chamber, volume of test chambers, or when samples should be taken for determining chemical concentration. Most of these issues are being addressed by two scientific groups. The American Society for Testing and Materials (Subcommittee on Safety to Man and the Environment, Committee on Pesticides) and the Interagency Regulatory Liaison Group are preparing standard methods. Methods being developed by these groups are improved over those currently available. Most recent drafts or published protocols from these groups offer the most valuable guidelines for a *Daphnia* life cycle test.

(2) *Calculations and Data Which Should be Recorded.* Useful information would include the initial loading (grams of organisms per liter), percentage survival and daily production of young for each treatment and control, general observations of other effects or symptoms, and unusual occurrences affecting test conditions. For flow-through tests, a description of the toxicant delivery system and flow rate as average volume of test solution passing through each test chamber in 24 hours would be helpful.

(e) *References.*

Standard Methods for the Examination of Water and Wastewater. 1976. 14th ed. American Public Health Association. New York, p. 762.

Biesinger, K. E. 1975. Tentative procedure for *Daphnia magna* chronic tests in a standing and flowing system. FEDERAL REGISTER 40(123):26902-26903.

Winner, R. W. 1976. *Toxicity of Copper to Daphnids in Reconstituted and Natural Waters.* U.S. Environmental Protection Agency, EPA-600/3-76-051.

C-5.7. *Test 6: Mysidopsis bahia Life Cycle.* (a) *Objectives.* Reproduction and growth of invertebrates are important factors when evaluating adverse effects of chemical substances that will enter marine or estuarine environments. For example, Mysid shrimp (*Mysidopsis bahia*) represent a link between plankton on which they feed and fish which feed on them. The objective of this test is to give a preliminary indication of potential effects of a chemical substance on an important link in marine food chains.

(b) *Rationale. Mysidopsis bahia* is an excellent species for life cycle test for marine invertebrates due to its sensitivity to known toxic chemicals, ease of culturing, short life cycle, and importance in near-shore marine food webs. The combination of a well designed test methodology and the desirable qualities of *M. bahia* are unique at this time. No other life cycle test on marine invertebrates offers the same advantages.

(c) *Test Description.* Young *M. bahia*, less than 24 hours old, are exposed to various concentrations of a chemical substance. Survival and production of young are monitored daily for 28 days. The impact on the shrimp is determined by comparing reproduction and mortality of animals exposed to a chemical substance with those of controls.

(d) *Methods*—(1) *Test Details.* Nimmo, et al (1976) and Nimmo, et al (1978) give details for culturing and performing a life cycle test for *M. bahia.*

(2) *Calculations and Data which Should be Recorded.* The loading, percentage survival, and production of young in each treatment should be recorded, along with a summary of general observation of other effects or symptoms, and unusual occurrences affecting test conditions. For flow-through tests, a description of the chemical delivery system and flow rate as an average volume passing through each test chamber in 24 hours would be useful.

(e) *References.*

Nimmo, D. R., et al. 1976. *Aquatic Toxicity and Hazard Evaluation.* F. L. Mayer and J. L. Hamelink, eds. ASTM 635. p. 109-126.

Nimmo, D. R., et al. 1978. *Bioassay Procedures for the Ocean Disposal Permit Program.* EPA-600/9-78-010. pp. 59-60, and 64-68.

C-5.8. *Test 7: Fish Embryo Juvenile Test.* (a) *Objectives.* The objective of this test is to give preliminary indication of potential effects of a chemical on fish. Chemical substances can have significant chronic effects on individual fish at concentrations 2 to 500 times lower than LC_{50} values. When long term exposure is probable, data on chronic effects contribute to assessment. Any chemical substance, whether dissolved or suspended, predicted to continously enter natural waters is a prime candidate for chronic testing. If the chemical will primarily enter a freshwater system, freshwater species are most appropriate. Similarly, if an exposure is mainly marine or estuarine, test species from those habitats are the most appropriate.

(b) *Rationale.* A fish embryo-juvenile test usually provides data indicative of a chronic study. The test exposes early life stages of the fish, which are usually the most sensitive part of the life cycle of a fish. Differences in sensitivities between a chronic and embryo-juvenile test are generally small or negligible, while differences in cost are great. The embryo-juvenile test is usually an excellent, cost effective substitute for a chronic fish study.

(c) *Test Description.* In an embryo-juvenile test, fish eggs and fry hatched from these eggs are exposed to a chemical at several concentrations for a few weeks. Effects on hatchability of eggs, and growth and survival of fry are determined by comparing responses at each concentration with the control.

(d) *Methods*—(1) *Test Details.* Acceptable methods may be found in Allison and Hermanutz (1977), Eaton et al. (1978), Hansen et al. (1978), and Sauter et al. (1976). Some of these references are for the life cycle or partial life cycle tests. Duration of these tests may be modified to include only the embryo-juvenile portion of the life-cycle (e.g., for sheepshead minnows, 28 days).

(2) *Calculations and Data Which Should be Recorded.* Percent hatch, percent survival, and growth as a percentage of control fish growth along with 95% confidence limits should be recorded for each treatment and control. Other useful information includes general observations of any behavioral or physical abnormalities, the loading and unusual occurrences affecting test conditions. For flow-through systems, a description of the test chemical delivery system and flow rate as average volume of test solution passing through each test chamber in 24 hours should be recorded.

(e) *References.*

Allison, D. T. and R. O. Hemanutz. 1977. *Toxicity of Diazinon to Brook Trout and Fathead Minnows.* U.S. Environmental Protection Agency, EPA Series 600/3-77-060.

Eaton, J. G., J. M. McKim and G. W. Holcombe. 1978. Metal toxicity to embryos and larvae of seven freshwater fish species. I. Cadmium. *Bull. Environ. Contam. Toxicol.* 19:95-103.

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C-5.9 *Test 8: Fish Bioconcentration.* (a) *Objectives.* Bioconcentration, the uptake of a compound from water into living tissue, affects the movement, distribution and toxicity of chemical substances in the environment. A substances that bioconcentrates may affect life far removed from the initial points of environmental release and may alter ecological processes at concentrations much lower than predicted from acute and subacute results. Bioconcentration is the first step in the process of food chain biomagnification; therefore, substances that bioconcentrate should also be evaluated for their effects on organisms other than fish.

Results of bioconcentration studies are useful in assessing risk to the environment especially when the substance is highly lipid soluble (e.g., octanol/water partition coefficient is

greater than 1000), poorly soluble in water (e.g., water solubility less than 0.9×10^{-3} molar at 20°C), and does not undergo rapid chemical or biological transformation. At this time, a persistence standard, i.e., the concentration below which a chemical poses only a minimal hazard to the environment, is not available. Such a standard would need to reflect the local variability in the chemical and biotic characteristics of aquatic environments.

(b) *Rationale.* Since a standardized bioconcentration test is not currently available, the most frequently used method for measuring bioconcentration is recommended. This procedure is based on a steady state approach. At this time, there is insufficient data available to conclude that any other approach (e.g. kinetic approach) is superior.

Since adsorption of hydrophobic chemicals is a physical process that should not vary significantly between organisms, the choice of test organism, i.e., fish, is based on sampling convenience, ease of culture and handling.

(c) *Test Description.* Fish are exposed to the chemical substance in water for 28 days or until their tissue concentration reaches steady state. After steady state or 28 days, fish are placed in uncontaminated water for 7 days. During the exposure (1-28 days or steady-state) and depuration (1-7 days) periods, fish are sacrificed periodically and the concentration of chemical substance in their tissues is measured. The bioconcentration factor, the relative uptake rate, and the depuration rate constants are estimated from these data.

(d) *Methods*—(1) *Test Details.* The most commonly tested species are fathead minnow, *Pimephales promelas*, and bluegill sunfish, *Lepomis macrochirus*. Examples of other acceptable test species and handling, holding, and acclimation procedures are given in EPA (1975).

The exposure should be conducted in 60 liters or more of water in a glass aquarium. The chemical should be delivered with a conventional Mount-Brungs diluter (Mount and Brungs 1967), modified to give only one concentration of chemical, or a metering pump with diluent water pump. Both delivery systems discharge directly into the treated aquarium. Diluent water should be delivered at a rate sufficient to maintain the oxygen concentration greater than 60% of saturation. The final concentration of the test chemical should be $\frac{1}{10}$ to $\frac{1}{100}$ of the 96 hour LC_{50}, or the highest concentration not having a toxic effect during the test. No observable toxic effects should be evident in test animals at any time during the test. Water temperature should be 22±1°C, and photoperiod should be $\frac{12}{12}$ hours.

The test substance should be added to the dilution water without use of any solvents or carriers other than water, if possible. If carriers or solvents other than water are necessary, they should be kept to a minimum. Hydrochloric acid may help prepare aqueous stock solutions, but it can affect the pH of the test solutions appreciably. Dimethylformamide, ethanol, methanol, and triethylene glycol are recommended for use in preparing stock solutions of water insoluble chemicals. The solubility of the test chemical in the stock solution should be determined. If a carrier is used, the experimental design should contain appropriate controls to determine any carrier effects on the results.

One day prior to testing, 80 acclimated and sexually immature fish of uniform size should be placed in a control tank and the diluent system for the treated aquarium started. Fish should be large enough for ease of sample preparation, but should not mature sexually during the test because mature females often accumulate more test chemical than males. After verification of the desired concentration of test chemical in the treated tank, 65 of the fish should be transfered to the test tank and 15 fish left in the control tank. Five control fish are analyzed on day one and the last day of exposure and at the end of the test. The fish should be fed throughout the test; however, feeding on sampling days should follow sampling of the fish.

The concentration of the chemical in water should be determined daily by analytical methods that yield greater than 90% recovery of the test chemical. To find the degree of bioconcentration of the test chemical, five fish should be removed after 1, 2, 3, 4, 7, 14, 21, and 28 days of exposure and the test chemical concentration and sex (if obvious) of each whole fish determined. Exposure can be terminated early if the concentration of test chemical in fish reaches steady state. After steady state or 28 days, the remaining fish should be placed in an aquarium containing water free of test chemical. This begins the depuration phase. Five fish should be removed 1, 2, 4 and 7 days later and the test chemical concentration and sex (if obvious) for each fish determined. These sampling days and fish numbers are minimal; additional and more frequent sampling is desirable. Analytical methods should recover greater than 90% of the chemical from tissue, as demonstrated by recovery studies. Flow-through waste water should be treated with particulate filters and activated charcoal or other suitable means to remove the test chemical, impurities or other degradation products before discharging the water.

The mean concentration of chemical in water should be recorded each day that fish are collected, and the bioconcentration factor calculated by dividing the concentration in tissue by the average measured concentration in water up to and including the day sampled. Bioconcentration factors vs. time should be plotted to determine if a steady-state was reached, and if so, the steady state value is recorded as the bioconcentration factor. If steady-state is not reached, the data should be analyzed using a curve-fitting technique to estimate the steady-state bioconcentration factor.

(2) *Calculations and Data Which Should be Recorded.* The bioconcentration factor, the uptake rate constant, and depuration rate constant should be calculated for the test chemical and significant impurities, depending on the quality of the data. Other useful information includes the mean (±SE) concentration of the test chemical and significant impurities in both tissue and water, for each sample day, the concentration of any solvents used in preparing test solutions, behavioral and physiological abnormalities observed and unusual occurrences affecting test conditions.

(e) *References.*

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